

# Genes, patents and sex determination

**A British regulatory committee has made a fool of itself by elevating a service for sex determination offered by a London clinic to an occasion for a debate on novel "serious ethical questions".**

THE danger that the business of new human biology, genetics included, would get out of hand has been mounting for several years. Unwittingly, the research community's incorrigible habit of deepening understanding has been responsible for a good part of the trouble. There would not, after all, have been two decades of public alarm about genetic engineering if people had not learned to unstitch and rejoin pieces of DNA. Administrators and businessmen have also helped to muddy the field, as when US officials decided to seek patents for segments of genes whose function is not known. More serious issues still are thrown up by the way in which laboratory techniques now commonplace in molecular biology laboratories have been legitimately patented, with consequences for research that may be as serious as a patent on the use of water as a solvent for polar substances, and which may also cramp genetic diagnosis by physicians (see page 285). Yet there is now also a danger that those whose professional concern is to regulate the uses made of new techniques, recommending legislation when they deem it necessary, have become part of the problem, not the solution.

So much is clear from the reaction of the British Committee on Human Fertilization and Embryology, and in particular of its chairman, Professor Colin Campbell, to the revelation that a clinic in north London offers as a service a means of determining the sex of children not merely unborn but not yet conceived. The committee brought forward the publication of a discussion paper listing the pros and cons of sex determination, while its chairman accompanied the launch with the opinion that sex determination raises "serious ethical questions". Bishops and archbishops have since been free with comments to the British press.

Sadly, the concatenation of these two events has placed the issue of sex determination on the wrong agenda. As with all new medical techniques, there is a danger that unscrupulous people may exploit the wish of others to be cured or improved; now that the medical profession is no longer the sole arbiter of what is permitted, there is a case for consumer protection agencies taking a close interest in the claims of those who offer services not yet part of orthodox medicine. But at this stage it is ridiculous that so much should be made of a technique widely practised elsewhere and whose social consequences are likely to be only marginal.

Where pregnancies are concerned, unenviable Britain should be most concerned with the large proportion that are apparently unpremeditated, witness the high abortion rate and the habit of single motherhood. There are also still too

many people unaware that pregnancy is a choice or, knowing that, are constrained from making it. But the idea that the sex ratio of the British population could be substantially affected by the mass-marketing of what is now offered is absurd. It would be different if there were a method entailing no external interference, but nothing of that sort is on the cards.

The committee's dilemma, therefore, boils down to the question whether its title and its terms of reference require it to act on, and perhaps against, every single innovation seeming to come within its remit. Its eagerness to do so confirms the general view that the British are not libertarians. But the same committee has serious work to do, adjudicating on licence applications from researchers bent on innovation in human embryology. It can only weaken its authority if it regards every mildly relevant article in a newspaper as a pointer to new "serious ethical questions". □

## Policy by default

**The British government will soon have an energy policy thrust upon it.**

Mr John Major's accident-prone government is likely to pay a high price for its insensitive decision last October that 31 of 50 remaining British collieries would be shut and 30,000 miners thrown out of work: it will have an energy policy thrust down its throat. That is the most probable outcome of the interdepartmental search for a more palatable policy on coal. Since Mrs Margaret Thatcher (as she then was) was first elected, the British orthodoxy has been that there is no need for an energy policy, with all the familiar uncertainties about future demand and the cost of future supply, but that competing fuels should find their competitive level in a free market. That is the spirit in which the gas and electricity industries, once nationalized, were privatized. The still-nationalized coal industry, called British Coal, was to have been next in line, although that prospect has now receded. What the government has now been forced to recognize is that the market is far from free.

The fuss about the miners is one sign of an imperfect market, if in that case the external constraints are political. The general indignation at last year's announcement had several roots. One was the belief that such an abrupt announcement was unfair to those who would lose their jobs (and the courts have since confirmed that British Coal would



have broken the government's own labour legislation if it had gone ahead). Another was the conviction that the timing of the decision was a folly, when unemployment was rising rapidly (as it still is) towards three million. Yet another was the conviction of many politicians that the government was dealing badly with the miners who had kept working through the bruising miners' strike in 1984–85. Miners themselves were naturally moved by the immediate threat to their jobs, of which they are traditionally inordinately fond.

Whatever the economic or social validity of these views, they are political realities and, thus, causes of market imperfection. One way or another, perhaps by direct subsidy, the government will now be compelled to tilt the economic balance towards coal. But this is not the first time that the British energy market has been biased by external considerations. During the privatization of the electricity industry, the Treasury's insistence that electricity sales should provide for the eventual dismantling of British nuclear plants led, first, to the decision that the nuclear plants should remain nationalized and, second, that there should be a levy on all British sales of electricity to provide for decommissioning. That arrangement tilts the balance in favour of nuclear plants, which the government then rightly considered to be strategically important. A similar decision has been made on windmills, which have been given favourable terms of access to the national distribution grid.

So what will be the outcome of the latest further proof that the market is imperfect? Much hangs on how the government's freedom is constrained by international obligations, not least towards its partners in the European Communities, but a subsidy for coal is on the cards. That will be justifiable if it helps to tide the miners over an uncomfortable patch, five years or so, but it cannot in the long run be a device for keeping collieries at work: the labour content in coal is greater than in other fuels, with the consequence that its cost must rise more quickly than the general inflation of salaries. That, and not the quantity of coal left in the ground, is why coalmining must be a declining trade in countries whose prosperity is increasing. The best assurance of the future of British coalmining would be declining prosperity, which even the miners cannot wish for.

The big danger in the government's internal review is that the outcome will be a further polarization between coal and nuclear energy. Some are already suggesting trading the nuclear levy for a subsidy for coal. But that makes no sense. The principle that the costs of operating nuclear plants should include some allowance for decommissioning is impeccable; "internalizing the externalities" is how economists of the environment describe the process. But present estimates of cost are probably unreasonably high, and would be more so if plants were designed with that end in view. If, even with better estimates, nuclear plants after that now under construction at Sizewell in Suffolk are uneconomic, they should not be built.

But would that not mean that Britain would permanently give up its strategic interest in civil nuclear power? Under present arrangements, yes, but only because the government has virtually washed its hands of publicly financed research

in the field. The lesson of the past few uncomfortable months is that the energy market must necessarily be compromised by long-term considerations: the uselessness of disused coalmines and the need to anticipate a future in which nuclear power must be an important source of power. Without breaching European agreements, the government could sensibly, and in the public interest, keep these options open by imaginative research. It may seem a further climb-down to be sponsoring reactor research again, but in logic there is no choice. □

## Education for refugees

**The University of Warsaw has made imaginative provision for students in the former Yugoslavia.**

PEOPLE wring their hands over the flood of refugees between and from the parts of dismembered Yugoslavia. Reactions range from regret, perhaps tinged with genuine compassion, to the fear that this comparatively minor wave in this decade's inevitable tide of migration may be a threat to the jobs, or the civility, of those who work and live elsewhere in Europe. In the midst of the recession, the general opinion of those turned into refugees by an interlocking series of unusually cruel civil wars is that they constitute an embarrassment, more a threat than a spur to economic renewal. Hungary and Germany have so far borne the brunt of numbers, but without enthusiasm, perhaps forgetful that the successive waves of migration to the United States in the nineteenth century eventually proved to be an economic benefit.

The senate of the University of Warsaw has now provided the rest of us with a reminder that the people displaced from what was Yugoslavia constitute more than an undifferentiated crowd whose only meaningful characteristic is a head-count. The crowd, of course, includes young people of ability who would, without the civil wars, have expected to go to some university in September. What will happen to them now that they live in refugee camps instead? Warsaw offers a practical solution: scholarships to five young people who have had to leave their home countries in what was Yugoslavia. Recognizing that five is a small number, the rector of the university, Dr Andrzej Kajetan Wróblewski, says in a letter that "we wish to be able to do more, but our resources are very limited".

Wróblewski goes on to plead that other universities and academic institutions should follow Warsaw's lead, and he is right. The disintegration of Yugoslavia has been a shock for the rest of Europe not simply because of the immediate military dangers, but because of the likelihood that the present turbulence will drag on for decades. What better insurance against that prospect than that something should be done to care for the education of Yugoslavia's young people, in whose hands some part of the future will rest? And in what better way can academics and their institutions, now fretting with frustration over what was Yugoslavia, help to shake off that debilitating state of mind? □

# Mixed reaction greets new gene patent proposals from Brussels

**London.** Responding to growing public concern, the commission of the European Communities (EC) has revised its thinking on the protection of biotechnology inventions. In particular, the commission wants member countries to consider ethical questions in granting patents on living animals, and to exempt farmers from paying licence fees on genetically engineered seeds grown for their own purposes from purchased stocks. The commission has also proposed that no patents be issued on genes (and thus, by implication, on fragments of genes) whose biological function is unknown.

Despite reservations from some quarters, the second and third of these proposals are likely to find widespread support. Farmers throughout Europe have sought an exemption from rules that would undermine what has traditionally been considered a 'farmer's privilege', namely the right to produce seeds from one crop to sow on the next year. Scientists have strongly opposed actions by the National Institutes of Health to obtain patents on lengths of DNA sequences even though their function is not known; such a move, they assert, could undermine the Human Genome Project.

The fate of the first proposal is less predictable. Although animal-rights groups say that it improves on earlier proposals by the commission, many claim that the language is still inadequate. In particular, they argue that only a minimal demonstration of the human value of a transgenic animal would be needed for a patent.

The new directive, now being circulated, would bring into line the existing patent policies of the EC's 12 member states. The first version of the directive, published in October 1988, was widely considered to favour the biotechnology industry.

The revised proposals follow stormy debate in the European Parliament, where support was strong for the 'farmers' rights' exemption. There has also been widespread controversy over the decision by the European Patent Office to reject moral arguments against the patenting of Harvard University's Oncomouse (see *Nature* 361, 103; 1993).

In revising its original proposal for a directive, the Commission has included a paragraph that would ban three types of inventions or discoveries. For example, it states explicitly that no patents can be issued on the human body "or parts of the human

body *per se*". This would not exclude patents on parts of the body (for example cell lines) capable of functioning outside the body. In contrast, however, the directive adds that "it goes without saying" that a patent could not be granted on, for example, "a human gene neither the function of which nor the protein for which it codes is known".

ing the genetic identity of animals which are likely to inflict suffering or physical handicaps without any benefit to man or animal".

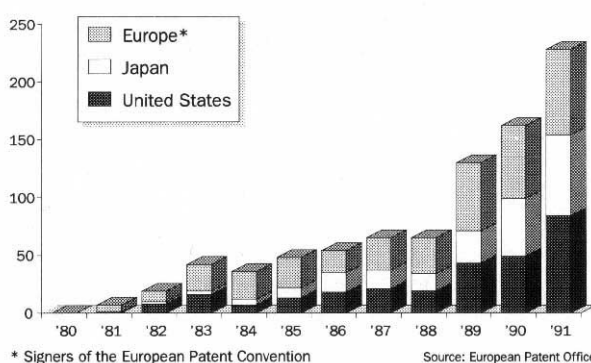
Commission officials believe that patent law is not the appropriate means to protect animals used in research. They say that this paragraph, patterned on a clause in the European Patent Convention that precludes patents on inventions considered to be against public morality, means that a patent cannot be issued on an invention which inflicts suffering on an animal "unless it may be beneficial [to humans]".

Animal rights groups say that this wording remains an unacceptable compromise. "I am very disappointed", says Peter Stevenson, research director of Compassion in World Farming, one of the groups that organized a protest earlier this month. "On first look, it appears to retain the morality clause of the European Patent Convention. But from a legal point of view, including the phrase 'without benefit to man or animal' makes everything else worthless, since all that an applicant would have to do would be demonstrate a potential benefit to man."

At present, EC member states are gathering comments on the directive before deciding how to proceed. Continued protests from animal rights groups are likely to resurface when the new directive is discussed at the Council of Ministers next month — particularly because the presidency of the Council is held by Denmark, home to some of the most vocal protests.

Commission officials hope that the new directives will be adopted by the Council of Ministers and become law within nine months. On past experience, this timing seems highly optimistic. **David Dickson**

## Who gets European biotechnology patents?



British patent experts say that this restriction, if adopted, would prohibit patents on cDNA fragments of unknown function.

The second proposed exclusion would be on processes for modifying the genetic identity of the human body "for a non-therapeutic purpose which is contrary to the dignity of man". Commission officials explain that the language has been carefully chosen to provide protection against what they describe as the "spectre of eugenics", while at the same time allowing patents to be granted for somatic gene therapy.

There is also language, completely missing from the earlier draft, stating that patents cannot be granted on "processes for modify-

## UK gene therapy gets go-ahead

**London.** The British government this week is expected to give the green light to gene therapy experiments by announcing the creation of a new review board to oversee all such experiments. The work of the main board will be supported by advice from specialist committees set up to consider technical aspects of the research involved.

The long-awaited decision is based on the recommendations last January of a committee headed by Sir Cecil Clothier that concluded that there was no ethical objections to somatic gene therapy, but suggested a moratorium on germline therapy.

Although there is not expected to be a rush to carry out gene therapy experiments — some British researchers claim that the medical profession is much more conservative in its approach to new disease treatments than its counterpart in the United States — several research teams are still keen to start work. In particular, protocols have been prepared for research into the possible treatment for cystic fibrosis sufferers, and for research on transplanting bone marrow cells containing a manipulated adenosine deaminase (ADA) gene.

**David Dickson**



# New EC commissioner is seen as friend of basic research

**Brussels.** The arrival of Antonio Ruberti as the new research commissioner for the European Communities (EC) has raised hopes that basic research will have a higher profile in the EC's forthcoming Framework programme. He would like by June to win approval for the five-year ECU14.7 billion (US\$19 billion) programme, which begins in 1994, and then turn towards persuading member countries to take more of a 'bottoms-up' approach to research projects.

Ruberti, 66, has an appropriate background for the job. An electronics engineer



Ruberti works on Framework.

by training, he was rector of the University of Rome for more than a decade before becoming Italy's Minister for Research in 1987. Losing that post after last spring's general elections, he won a seat in the Italian Parliament for the first time as a candidate of the Socialist Party.

In a country fraught with political connivance and corruption, Ruberti emerged from government service with his integrity untainted and with the respect of the scientific community. His main achievement was to unite research and universities under a single ministry, allowing him to raise the national profile of research and to win greater autonomy for the notoriously overcentralized university system. He was responsible for the creation of the Italian Space Agency (ASI) and for many major national research programmes, including the Antarctic programme.

Ruberti's arrival, which reportedly has already brought a friendlier style to the way the commission operates, coincides with an organizational shakeup that includes merg-

ing education with research. He believes that the new arrangement will lead to greater exchanges among faculty now that Europe is an open market for professors. "It is an important experiment to put education and research together", he says.

The commission is waiting to see how far Ruberti will go in reversing the legacy of his predecessor, Filippo Pandolfi, a non-scientist who was criticized for pushing EC research too far towards industry at the expense of basic research. Ruberti believes that the EC should have much closer links to, but not overlap with, the independent Eureka programme which undertakes to promote industrial research throughout the EC countries and beyond. Although he plans to go forward with the Framework programme drawn up by Pandolfi, he says that he is not afraid of change and that some amendments are to be expected.

On the other hand, some fairly fundamental changes in procedures are promised. Ruberti is keen to give national research councils a larger voice in the directing of EC funds, and he strongly supports greater co-operation at all levels between countries. With such goals in mind, it is not surprising that Ruberti embraces the Human Capital and Mobility Programme, which sponsors the movement of young researchers within Europe. Infamous for its labyrinthine complexities, this programme is a major feature of Pandolfi's fourth Framework budget, and Ruberti is not unduly alarmed by complaints about its tortuous application procedures followed by incomprehensible delays in funding.

Those delays could worsen under the Maastricht Treaty, which requires major proposals to be approved by all three EC bodies — the Commission, the Council of Ministers and the European Parliament. At present the council receives advice but decides unilaterally. Ruberti believes that requiring codecisions will reduce delays. But staff at the commission do not share his optimism, believing that gaining the initial approval process will be quite lengthy.

Ruberti's research portfolio will be more restricted than that of Pandolfi's. The organizational changes have moved some of the more industrially relevant research, notably telecommunications and informatics, which make up 40 per cent of the budget, into the industrial commission, headed by Martin Bangemann, a former German economics minister.

Ruberti hopes that a first draft of the fourth Framework programme will be ready by the end of February and be approved before Denmark relinquishes the EC presidency in June.

Alison Abbott

# Lawyer named minister for German research

**Munich.** German Chancellor Helmut Kohl has named a strong advocate of linking science to economic development as the country's new research minister. Matthias Wissmann, a lawyer and former Christian Democrat economics spokesman, was last week chosen to succeed Heinz Riesenhuber, who has served in the post for more than a decade, as part of a modest government reshuffle.

Kohl explained his surprise move by saying that he wanted a younger cabinet (Wissmann is 43 and Riesenhuber 57) in the run-up to the 1994 elections and one with greater geographical diversity. But a more political motive may also underlie the decision: a desire to tie research policy more closely to economic policy. A chemist by training, Riesenhuber was seen as a champion of science who fiercely defended the budgets of both basic and applied research.

Wissmann believes in close links between "intelligent economic policy" and research politics. He is particularly supportive of the rebuilding of industrial research in the new eastern states and will fight for spending an additional DM150 million (US\$100 million) above the DM750 million already promised this year for specific applied research programmes in the former East Germany. As for Riesenhuber, Kohl has offered him a new — and only vaguely defined — role to "enhance scientific collaborations with the United States and Japan".

Alison Abbott

# Clinton lifts ban on fetal research

**Washington.** Two days after taking office, US President Bill Clinton overturned a ban on the federal government sponsoring research involving the use of fetal tissue in transplants. In overturning the prohibition, Clinton cited the promise of fetal tissue research in developing treatments for Parkinson's, Alzheimer's disease, diabetes and leukaemia.

The decision opens the way for researchers to apply for grants from the National Institutes of Health (NIH) and also is expected to free up other sources of funding. Eugene Redmond of the Yale University School of Medicine, who has relied on foundations and private individuals to support his work related to Parkinson's, says that foundations and drug companies that otherwise would have considered funding fetal tissue research during the Bush presidency declined because of the federal ban.

Tony Reichhardt

# Inquiry into misconduct excoriates Michigan State

Four years of acrimony at Michigan State University (MSU) have culminated in the finding that a graduate student was guilty of scientific misconduct for keeping research data to herself and publishing without the consent of the supervisor. Although an independent inquiry into the affair recommends that the student should be expelled, the chief casualty is likely to be the reputation of the university administration, which is accused of bias, inordinate delay in answering letters, buck-passing, a lack of understanding of the traditions of biomedical science and a failure to meet its responsibilities to the academic community. The inquiry recommends a review "to determine whether corrective actions are necessary".

The inquiry, whose report has not yet been published, was masterminded by Barbara Mishkin, a Washington lawyer specializing in misconduct. It was convened last March and submitted its report to the university on 14 December. The university has received comments from the principals and is expected to render a decision within a month. Its actions will be reviewed by the National Institutes of Health (NIH), which funded the research.

The trouble dates back to 1987, when Maie ElKassaby became a PhD student in the pathology department of the veterinary school and started work with Jeffrey Williams of the microbiology department, holder of an NIH grant to study parasitic worm diseases in collaboration with a group of researchers and physicians in the Sudan.

By the summer of 1989, Williams and his student had fallen out.

ElKassaby's membership in the PhD programme was terminated on two occasions, but by the end of 1989 the administration arranged that she should work for a master's degree under a new guidance committee. (She was reinstated to the PhD programme in December 1990.)

With the help of the academic committee, ElKassaby then prepared a manuscript for publication, but her offers of co-authorship to scientists at the Upjohn Company (who had developed her radioimmunoassay technique) and to physicians in the Sudan were declined because they had not seen the data on which the manuscript was based.

The inquiry says that these objections were "rejected or ignored" by the university administration and the guidance committee, one of whose members wrote a covering letter for the manuscript submitted to the *Tropical Medicine and Parasitology*. Ironically, the manuscript was accepted without review because of a "clerical error".

The inquiry finds that ElKassaby was guilty of scientific misconduct for having withheld data from her collaborators. Of ElKassaby's publication, the inquiry says that to have published work "inseparable from that of others" without their agreement or acknowledgement also constitutes "scientific misconduct", but that "her guilt is mitigated" by the connivance of her guidance committee and of MSU's vice president for research, John Cantlon.

The inquiry also exonerates Williams from ElKassaby's counter-charges that he sought to use her work for private gain, and that his termination of her research assistantship was improper. On the other hand, the inquiry accepts ElKassaby's view that Williams should not have disclosed her identity, but attributes that to the "collective inaction of the MSU administration".

The inquiry concluded that the university administration acted from fear that ElKassaby had been wrongly dismissed and that she might win a legal action, as well as by the suspicion that Williams was persecuting her. It complains that the administration should have "paid as much attention to faculty rights as to the rights of the student".

The charge that the administration made light of its "responsibilities to the academic community" is illustrated by its willingness to contemplate awarding ElKassaby a PhD "when her contribution to the research in question was minimal". MSU is also accused of having violated US federal regulations in carrying out its internal investigation of the dispute (which exonerated the academic guidance committee's members).

Apart from the recommendation that ElKassaby's PhD programme should be terminated and that she should be expelled, the inquiry says that MSU should develop policies for resolving conflicts between its departments and colleges, and for handling allegations of scientific misconduct. The final recommendation is that its report should be published.

John Maddox

## Montagnier and Mayor create AIDS foundation

**Paris.** Luc Montagnier of the Pasteur Institute and Federico Mayor, director general of UNESCO, have co-founded a private organization to fund non-mainstream AIDS research and to support AIDS-related educational and social projects in Africa. But it is not yet clear whether this "World Foundation for Research and Prevention of AIDS", unveiled on 28 January in Paris, will have sufficient resources or clout.

Montagnier hopes for international participation, although the only declared donation so far is US\$500,000 from an Italian bank. UNESCO itself is not directly involved in the venture. One of the first areas to be targeted is Montagnier's own research on mycoplasmas, leaving him open to charges of conflict of interest in his role as final arbiter of scientific activities.

The foundation expects to establish applied clinical research centres in Europe and Africa for those who are seropositive. The foundation also plans to establish prevention and social programmes in Africa; the first would provide FF5 million (US\$1 million) to educate orphans whose parents have died of AIDS.

Declan Butler

## Meetings on British science

*NATURE* is organizing two one-day public meetings to discuss proposals for the forthcoming white paper on the organization of British science, in Edinburgh and London.

On **Friday 19 February**, there will be a meeting in **Edinburgh**, jointly sponsored by the Edinburgh International Science Festival. The speakers will be Professor John Glover (University of Dundee), Sir Graham Hills, Professor J Maxwell Irvine (University of Aberdeen) and Professor David Wallace (University of Edinburgh). The meeting will be at the Queen Mother Conference Centre, Royal College of Physicians, 9 Queen Street, Edinburgh, and will start at 9.30 a.m.

Admission will be by ticket, free of charge, obtainable from Bruce Durie, Edinburgh International Science Festival, 1 Broughton Market, Edinburgh EN3 6NU. Coffee and tea will be provided. There will also be a sandwich lunch for those who want it, at a cost of £5: please send a cheque, made out to the Edinburgh International Science Festival, with your ticket application.

On **Friday 19 March**, there will be a meeting at 9:30 a.m. in **London** at the Royal Society, 6 Carlton House Terrace, SW1. The speakers will include Sir Eric Ash (Rector, Imperial College London), Professor Michael Brady (University of Oxford) and Sir Mark Richmond (Chairman, Science and Engineering Research Council).

Admission will be by ticket, free of charge, obtainable from Mary Sheehan, *Nature*, 4 Little Essex Street, London WC2R 3LF. Coffee and tea will be provided. There will also be a sandwich lunch for those who want it, at a cost of £5: please send a cheque, made out to *Nature*, with your ticket application.



# Japan is cool to request to help pay to run CERN

**Tokyo.** A recent suggestion by the head of the European Laboratory for Particle Physics (CERN) that Japan should contribute to the running costs of the Geneva-based laboratory has received a frosty reception.

Earlier this month, Carlo Rubbia, director-general of CERN, in a follow-up to two visits by CERN officials last year (see *Nature* **359**, 5; 1992), suggested that Japan should contribute 2.5 per cent of CERN's operating costs, or about SFr25 million (US\$18 million) a year, to compensate for the increasing number of Japanese users of CERN. Japan is not a member of CERN and does not contribute to its operating costs, although it has provided the laboratory with some equipment. In 1990, Rubbia hinted at such a request to physicists at Tokyo University.

In the past ten years, the number of registered Japanese users of CERN has increased eightfold (see figure). The Japanese presence is comparable to that of the Netherlands, a paying member of CERN, and larger than that of some other member nations such as Switzerland.

The United States, another non-member, has more users than Japan, but Rubbia says that its use of CERN is roughly balanced by comparable European access to US accelerator facilities. But few Europeans have been attracted to Japanese accelerators, and member nations of CERN are requesting that the balance be restored financially.

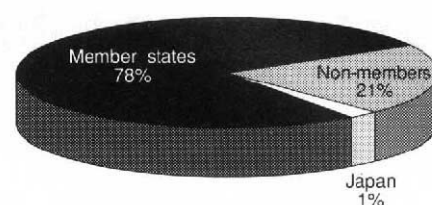
The 15-member accelerator subcommittee of Japan's Science Council, an advisory body to the Ministry of Education, Science and Culture, the ministry that funds Japan's participation in CERN, discussed Rubbia's

proposal last week at a meeting covering several issues, including the US Superconducting Super Collider (SSC). Many on the subcommittee, chaired by the former director-general of the National Laboratory for High Energy Physics (KEK) in Tsukuba, Tetsuji Nishikawa, feel that Rubbia's request is "sudden and unreasonable", resulting from CERN's recent decision to allow Germany to reduce its contribution to the laboratory because of the costs of German reunification (see *Nature* **360**, 701; 1992).

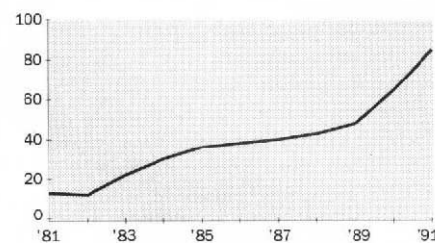
The subcommittee members say that paying to help run CERN would be unprecedented and would violate the principles of international collaboration in high-energy physics. They point out that operating costs in Japan do not include salaries, as they do at CERN, and that Japan pays the salaries of Japanese users. (However, the same is true of member nations who send scientists to Geneva.) Japanese users also point out that guidelines adopted in 1980 on interregional utilization of major facilities say that "operating laboratories should not require experimental groups to contribute to running costs". Japan believes that the number of Japanese users quoted by CERN overstates their participation, and points out that Japan has already contributed 30 per cent of the cost of the OPAL detector on the LEP electron-positron collider, which is used by Japanese scientists in Geneva.

Rubbia says that he would "much prefer" to reach a solution by evening up the exchange of scientists "rather than having money flow across borders". That would be made easier, he adds, if Japan proceeds with its plans to build a B-meson factory (see below). But some CERN member nations,

Status of CERN visiting scientists



Japanese usage has grown



Source: CERN

in particular Germany and France, are pressing for an immediate financial solution. The B-factory is "years down the road", says one European diplomat, who asks "how much longer are CERN member nations going to put up with people getting a free lunch?"

Another sticking point in the debate is Japan's role in funding the SSC. Europeans want to dissuade Japan from a joint consideration of their proposal and the SSC by emphasizing that they are seeking funds for Japan's current use of CERN and not for CERN's future Large Hadron Collider (LHC), a competitor of the SSC. But Japan believes the two issues are inter-related, and they do not plan to make a decision on CERN until they have an answer for US officials on their contribution to the SSC.

Akito Arima, president of Tokyo University and a member of the accelerator subcommittee, said in a letter to the subcommittee that a Japanese contribution to the SSC should be matched with one to CERN. "Everybody laughed", says one subcommittee member, "but I think everybody agreed".

In a related development from Washington, the Secretary of Energy in the Bush administration admitted to the US Congress shortly before leaving office last week that the department's campaign to attract foreign contributors to the SSC has fallen short of its goal. "Without a significant contribution from Japan", James Watkins wrote to US Representative George Brown (Democrat, California), "it is highly doubtful that the one-third foreign funding goal can be met." Watkins told Brown that only \$45 million of the planned \$250 million in non-federal contributions for next year has been obtained, part of a projected shortfall of \$1.3 billion by the SSC completion date in 1999.

David Swinbanks

## B-factory gets a boost

**Tokyo.** Plans to build a B-meson factory got a boost at last week's meeting of the accelerator subcommittee (see above). But the US Superconducting Super Collider (SSC) still casts a dark shadow over the future of the project.

The powerful subcommittee, which includes prominent professors from other fields outside high-energy physics, is supporting a proposal to build a B-meson factory at the National Laboratory for High Energy Physics (KEK) in Tsukuba rather than a plan to convert the main ring of the TRISTAN electron-positron collider at KEK into a gigantic synchrotron. The decision is a small victory for Japan's high-energy physicists, the majority of whom want the B-factory as Japan's next major project (see *Nature* **358**, 266; 1992). The synchrotron, with a beam energy of 10 GeV, would have been the most powerful in the world and an important tool for those who analyse and fabricate materials.

The subcommittee's decision, in theory, allows the Ministry of Education, Science and Culture to request funds for the project in the ministry's fiscal year 1994 budget to be submitted in August. Only one thing stands in the way — the SSC. If Japan decides in the next few months to put money into the SSC, the ministry could not accommodate both projects in next year's budget.

D.S.

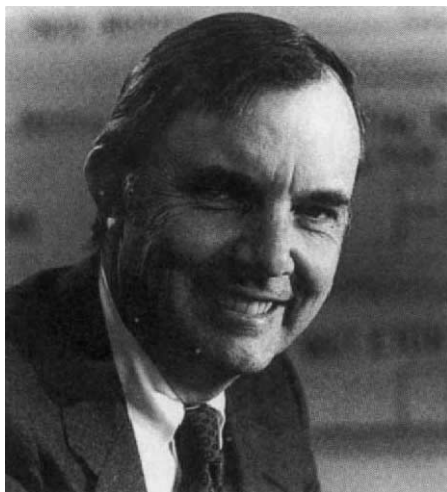
# NSF weighs value of supercomputer centres

**Washington.** An advisory panel is reviewing the rationale behind four supercomputer centres funded by the US National Science Foundation (NSF) as part of an overall look at the role of high-performance computing in science. A 14-member panel, meeting last week for the first time, discussed whether NSF should continue to pay for big, expensive mainframe machines at national centres in a world of cheap, powerful desktop workstations, high-speed communication networks and parallel processing. The group also will consider the appropriate relationship between NSF and other federal agencies with a greater investment in supercomputers.

The 14-member panel, appointed by NSF, is chaired by Lewis Branscomb of Harvard University and intends to meet several times before submitting a report in May to the National Science Board, which supervises the foundation's activities.

Last year, NSF spent \$56 million on the Cornell Theory Center at Cornell University, the National Center for Supercomputing Applications at the University of Illinois, the Pittsburgh Supercomputing Center at Carnegie Mellon University and the San Diego Supercomputer Center at the University of California, providing 56 per cent of their total budget. Formed in the mid-1980s, the centres are expected to be innovators, educators and proselytizers, finding new applications for supercomputers, attracting

new users and helping existing ones make the most of high-performance machines. But there is now concern that the centres in some cases serve mostly as 'cycle shops' for a small group of researchers who need large



**Branscomb looks at changing world.**

amounts of supercomputer time, or cycles.

The panel will look at more than just the fate of the NSF centres, however. "We have to think about how the whole relationship between the world of science and the world of computing has changed", says Branscomb, a former chief scientist at IBM. The panel

would like to anticipate high-end hardware and software that will be on the market in the next five years, as well as the uses to which they will be put.

Another question facing the panel is NSF's relationship to other public and private programmes. Federal agencies such as the Department of Defense, the National Aeronautics and Space Administration and the Department of Energy all spend more on supercomputers than does NSF and play a bigger part in the coordinated federal High-Performance Computing and Communications initiative begun last year to accelerate US progress in the field. Vice President Al Gore is expected to lead a campaign by the Clinton administration to strengthen the High-Performance Computing Act of 1991, which Gore introduced, as part of its pledge to invest in an information 'infrastructure'.

The panel is seeking the advice of the computer industry and other government agencies who use supercomputers and scientists, including professional societies. It also expects to consult with other state-run and university-based supercomputing centres, several of which have capabilities matching those of the NSF-supported centres. The panel includes researchers who have used high-performance computing in their own scientific and mathematical work — "people who have a good knowledge of the technology", says Branscomb.

**Tony Reichhardt**

## Institute is centre of political war in ex-Soviet republic

**Moscow.** An attempt by the newly independent Bashkortostan Republic to take over a research centre formerly operated by the Russian Academy of Sciences has dimmed prospects for researchers in the capital city of Ufa. A compromise has been struck to assuage the pride of local politicians, who formed a Bashkir Academy of Sciences as a way of asserting their independence from Russia, but many scientists have already left or begun to look for work outside the Ural Mountains region.

The problem started when the Bashkortostan Supreme Soviet decided to transfer authority for the Bashkir Scientific Centre, with its eight research institutes, to the newly formed Bashkir academy. But the academy is a purely national structure with little interest in fundamental research; three of its 14 institutes have nothing to do with science, and others are concerned with medicine, agriculture and other applied fields. The move was a blatant attempt to confiscate federal property, according to Henri Tolstikov, chairman of the Bashkir Scientific Centre, and was made without regard

to its impact on science.

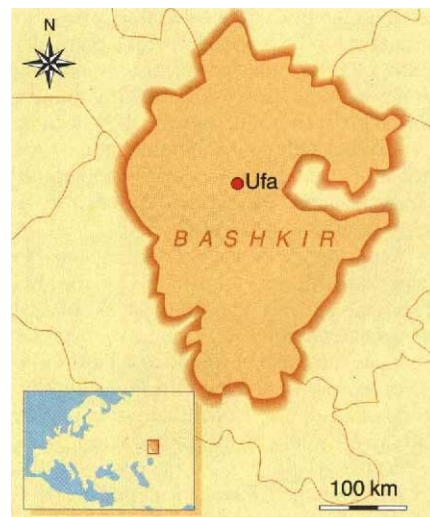
In response, Russia dispatched three vice premiers, including science minister Boris Saltykov, who strongly urged the Bashkortostan government to reconsider its decision. The discussions produced a compromise: the republic would drop its claim to the scientific centre if the Russian academy transferred control of the scientific centre from its Urals branch directly to the academy presidium.

Although this compromise is now in effect, there is concern that it will not hold. Tolstikov says that the ruling élite harbour nationalistic and anti-Russian feelings that could subvert the agreement and make it impossible for the Russian academy to exert control over the centre. Tolstikov has been branded an "enemy of the Bashkir people" for his refusal to go along with what he calls "the inflammation of sovereignties" spawned by the breakup of the Soviet empire.

In the meantime, the fate of academic science in the region hangs in the balance. Academician Nikolai Laverov, one of the members of the Russian delegation that

negotiated the compromise, insists that any attempt to seize academy property must be stopped. "Otherwise", he says, "we will be forced to declare the destruction of science in Bashkir."

**Vladimir Pokrovsky**





# Centocor staggered by poor clinical results

**Washington.** Centocor, Inc. of Malvern, Pennsylvania, suffered a serious setback last week when an interim analysis of the company's clinical data revealed an unexpectedly high number of deaths in a group of patients being treated with the company's leading product, Centoxin (or HA-1A). These adverse findings have forced Centocor, founded in 1979, to stop its phase III clinical trial in the United States and to suspend sales of the drug in Europe pending further analysis.

Centocor's share price plummeted after the announcement, falling 62 per cent to \$6.625. Many analysts now speculate that Centoxin will never be approved in the United States and that Centocor, at one time considered to be among the world's leading biotechnology companies, will be forced to cut its losses with Centoxin — a human monoclonal antibody designed for the treatment of Gram-negative sepsis and septic shock.

Centoxin is thought to act by binding specifically to the lipid-A component of endotoxin, the toxin released by Gram-negative bacteria. Company officials remain tight-lipped about the number of people who have died, saying that further analysis will be necessary to determine the higher mortality among patients treated with Centoxin who did not have Gram-negative bacteraemia (the group in whom Centoxin has no proven benefit) compared with patients in the same group who received a placebo.

"On the basis of our information to date, we do not believe that HA-1A is unsafe", says David Holveck, president and chief executive officer of Centocor. "The statistical requirement for a suspension of the US trial was conservative, but it was Centocor's responsibility to suspend all use of HA-1A if there was any hint of trouble", he says.

The best news for Centocor would be that it was a statistical anomaly caused by a flaw in the design of the clinical trial. However, if it turns out to be a problem with a particular batch of drugs or, worse still, something wrong with the drug itself, the drug may be finished. "Even if it is something that can be corrected, people remember that there was a problem, they don't necessarily remember the explanation", says David Stone, a stock analyst with Cowen and Co.

At the very least, Centocor is expected to dispense with its almost 200-person US salesforce. Jeffrey Casdin, a stock analyst with Oppenheimer & Co., says that the company may also be forced to sell off some of its excess manufacturing capacity (the company has three manufacturing plants) and lay off many of its clinical and regulatory staff.

Although there is an urgent medical need for new therapies against sepsis (there are

about 400,000 cases a year in the United States and the numbers are increasing), some clinicians have questioned the effectiveness of Centoxin and Xomen-E5, a rival mouse monoclonal antibody product produced by Xoma Corporation of Berkeley, California.

The warning signs concerning Centoxin appeared in 1991 when several letters critical of Centocor's earlier clinical trial data on Centoxin were published (*New Engl. J. Med.* **325**, 279–283; 1991). An increase in mortality among patients without Gram-negative bacteraemia in the group receiving Centoxin was also evident in Centocor's earlier phase III clinical trial (*New Engl. J. Med.* **324**, 429–436; 1991), according to Craig Tanio and Harold Feldman of the University of Pennsylvania. They wrote that this trend toward increased mortality in this group of patients "raises the question of whether HA-1A may be seriously toxic in a large proportion of patients presenting with sepsis".

Other clinicians argued that lack of a quick diagnostic test to differentiate between Gram-negative and Gram-positive infections would result in the use of Centoxin

in many patients for whom it has no proven benefit. Some analysts argue that the writing was on the wall for Centocor last April when the US Food and Drug Administration told the company that there was insufficient evidence to establish the efficacy of Centoxin and that a further phase III clinical trial must be undertaken.

Investors became disenchanted and the company's stock price dropped. Some investors were wooed back when, in July, Eli Lilly & Co. of Indianapolis, Indiana, invested \$100 million in return for the distribution rights to Centoxin and an option to obtain the rights to CentoRx, a cardiovascular agent.

Even if the US clinical trial was to be resumed, some analysts feel that the anti-endotoxin approach being taken by Centocor and Xoma could lose out to more broadly acting therapies that do not rely on knowing that the patient's infection is due to Gram-negative or Gram-positive bacteria. Antril, a recombinant interleukin-1 receptor antagonist being developed by Synergen, Inc. of Boulder, Colorado, appears to work against both types of infections. **Diane Gershon**

## WHO vote upsets Western nations

**Tokyo, Washington & London.** A sense of gloom has descended on the Geneva headquarters of the World Health Organization (WHO) following the reappointment last week of Japan's Hiroshi Nakajima as director general. The United States and European nations lobbied hard to replace Nakajima, whose management they have strongly criticized. Japanese officials, on the other hand, are elated because they see his re-election as an example of the country's growing presence in the international arena.

The 64-year-old pharmacologist, who joined WHO in 1973 and was appointed director general in 1988, is seen as having provoked the departure in 1990 of Jonathan Mann, the highly respected leader of WHO's AIDS programme (see *Nature* **344**, 283; 1990). And Donald Henderson, associate director for life sciences at the US Office of Science and Technology Policy and a former WHO official, says there has been "no vision and no progress" during Nakajima's first term.

Nakajima won last week's election by a vote of 18–13 over Mohammed Abdelmoumene of Algeria, the former deputy director general of WHO. In yet another example of what Western officials see as Nakajima's poor management style, Nakajima fired Abdelmoumene last August for "disloyalty" when he discovered the Algerian was running against him.

Japan put heavy pressure on developing nations to support Nakajima's re-election.

In December, the *New York Times* cited an internal US State Department report saying that the Japanese government had threatened to slash imports of coffee from Jamaica and marine products from the Maldives if they did not vote for Nakajima. Another rumour circulated that Japan would reduce its large voluntary contribution to WHO, now \$14 million a year, if Nakajima were defeated. These accusations were flatly denied by Japanese foreign ministry officials, who in turn saw Western opposition to Nakajima as "racist".

Japan's foreign minister, Michio Watanabe, welcomed Nakajima's reappointment and Yuya Niwa, Minister of Health and Welfare, attributed the result to "the fair evaluation of Mr Nakajima's ability and performance as director general".

But while Japanese officials celebrate, the mood in Geneva and Western capitals is grim. "Nakajima's management style threatens to derail WHO's main function of providing an international umbrella for important joint projects among countries that would be very difficult to pull off on a bilateral basis", says Henderson. British medical scientists are concerned that Western governments may withdraw their support for WHO's research programmes much as they did with UNESCO in the 1980s after continued criticism of its management.

**David Swinbanks, Jeffrey Mervis & David Dickson**

# Indians protest against US-led gene bank

**New Delhi.** The ransacking last month of the office of the Indian subsidiary of a US seed company has revived the controversy over a US-Indian project to build a modern seed gene bank and highlighted the often tense relationship between multinational companies and their hosts.

On 29 December, 100 members of a local farmers' association in the southern state of Karnataka broke into the Bangalore office of Cargill Seeds India, a subsidiary of Cargill Inc. of Minneapolis, Minnesota, destroying files and assaulting two Cargill employees. The mob left a note demanding a ban on the entry of multinational companies into the seed business. The raid was led by a retired local agricultural scientist, M.D. Nanjundaiya, and supported by a voluntary organization called the Gene Campaign headed by Suman Sahai, a professor of genetics at the University of Heidelberg.

The protesters fear that Indian farmers will become dependent on the companies for seeds developed from native plants and that, according to Nanjundaiya, "our scientists will be paying hefty royalties to get their own genes back". India opened its doors five years ago to foreign seed companies, and the Gene Campaign has asked 11 of them, including Cargill, to leave the country. "We have resolved to fight for the farmers' right to their genetic resources so that they can manage their own production

systems as they have done for hundreds of years", says Sahai.

US and Cargill officials are puzzled by such rhetoric. "We have not patented any Indian gene pool material", says Greg Lauser, a spokesman in Minneapolis for Cargill. The subsidiary in India was formed in 1988 to develop and sell seeds that have been adapted to local conditions, he says, and none of the seed produced in India is exported. Cargill has developed new hybrids of sunflowers, corn and sorghum.

Historically, India has shown little respect for the intellectual property rights of foreign inventors. Only recently, for example, has the government allowed outside seed companies to retain proprietary control over their own material. "I find it particularly ironic that this criticism is coming from a country that does not recognize the rights of others", says Joel Cohen of the US Agency for International Development (AID).

AID is paying 60 per cent of the cost of the \$24-million gene bank now under construction on the campus of the Indian Agricultural Research Institute in New Delhi. When completed in 1994, the gene bank will be able to hold as many as 800,000 accessions and the country will for the first time have the capacity for in vitro, long-term storage of rare and valuable seed lines. Its present system, operated by the National Bureau for Plant Genetic Resources, is al-

ready considered the third-best in the world, behind the US facility in Fort Collins, Colorado, and the Vavilov Institute in St Petersburg.

Despite the obvious improvement in the country's ability to preserve its genetic heritage, some Indian scientists regard the rules under which the new facility will operate as a throwback to the colonial era. "This is a totally one-sided deal", says Vandana Shiva, director of the Research Foundation for Science, Technology and Natural Resource Policy in New Delhi. "While western companies are patenting the contents of their seed banks, India was agreeing to provide open access to its own bank. All that India gets in return are a few seeds of jojoba [an oil-bearing desert plant] and sunflowers."

The Indian government says that the agreement preserves the rights of farmers and does not relinquish control of the country's genetic resources. India already participates in a global system that sends and receives germplasm, says Rai Singh Rana, director of the national plant genetics bureau, and the US contribution will strengthen India's research programme by providing additional equipment, training and opportunities for collaboration. The genetic characteristics of every seed in the gene bank will be recorded, he adds, ensuring that the origins of any new variety patented can be readily determined. **K.S. Jayaraman**

## Licences sought from PCR users in Britain

**London.** Genetic-screening services throughout Britain could have their costs increase significantly as a result of claims for royalties on one of the basic laboratory techniques used in diagnostic testing, polymerase chain reaction (PCR). This follows the granting over the past few weeks of two patents on the PCR process by the European Patent Office.

The UK Department of Health and the Swiss manufacturer, Hoffmann-La Roche, which holds the patent rights to PCR, are discussing the terms under which hospital laboratories will be permitted to use the technique for diagnostic purposes. The company has told the department that it would like institutions to have licences permitting them to use PCR, a procedure it has adopted with private companies. However, the company is also prepared to negotiate an umbrella licence with the department covering all nonprofit institutions.

Responding to concerns that the licensing fees being demanded by La Roche — which would add about 12 per cent to the cost of each screening test — will be a burden to screening programmes being carried out by the National Health Service, the

company says that these fees are intended to reflect standard practice in other countries.

"We perceive the British Health Service as a commercial activity, since it is paid for by taxpayers. You cannot say that it is free of charge", says Claude Montandon, director of licensing for Hoffman-La Roche in Basel. If an umbrella licence is agreed to with the health service, he adds, it would be based on an assessment of the "fair market value" of the tests.

The PCR technique, which is used to amplify a short length of DNA allowing it to be characterized, was developed in the early 1980s by Kary Mullis, working at the Cetus Corporation in California. It has become widely used in laboratories around the world.

The rights to PCR were bought by La Roche in 1991 from Cetus for \$300 million. Last year, following widespread concern in the research community about the cost of US licences, the company significantly reduced the price (see *Nature* **355**, 379; 1992).

The company has recently demonstrated that it intends to pursue vigorously those thought to be infringing on its patent rights. At the top of its list are companies which it claims are producing and selling TAQ

polymerase — the main enzyme used in the PCR reaction, which is covered by a separate patent — either without a licence or under a licensing agreement that should in principle restrict the sale to non-PCR uses.

The company is concerned that many scientists are buying TAQ polymerase from cheaper sources than the Perkin-Elmer Corporation, which has the sole rights to the sale of TAQ-polymerase for PCR. For example, it has recently sued the Promega Corporation, of Madison, Wisconsin, for breaching an agreement between the two companies under which Promega was permitted to sell TAQ polymerase only to those not using it for PCR.

More difficult for the company to police has been the use of machines other than those produced by Perkin-Elmer for carrying out PCR. The new patent rights awarded to La Roche by the European Patent Office mean that the company can now require anyone using such a machine to carry out PCR to buy a licence. It is this right which underlies the company's current demands on PCR users to enter into licensing agreements.

**David Dickson**



# Popper, Wolpert and critics

SIR — Lewis Wolpert (*Nature* 360, 204; 1992) seems so profoundly to misunderstand Popper's philosophy that I can only conclude he has never read any.

Popper does not maintain, as Wolpert claims, that "... only falsification is important. ..." in the procession of science. Popper proposed that falsifiability is the criterion for the demarcation between science and nonsense. However, a nonscientific or metaphysical theory can prove very influential in the procession of science, for example Popper's theory of falsifiability. The fact that Popper's theory is metaphysical seems to be the root of Wolpert's misunderstanding. A metaphysical theory can, it is true, be meaningful and useful, but if there is no means of testing its truth there can be no empirical support for that truth: that is why it is nonscientific or metaphysical by Popper's criterion. Hence, when Wolpert asks of Popper's theory, "How would one falsify it?", he does not, as one assumes he intends, point out that Popper's theory is nonsense, but demonstrates his own misunderstanding. Wolpert seems to hold the misconception of falsifiability not as the demarcation between science and nonsense but between sense and nonsense. Thus, Wolpert suggests that the nonsense statement, "... eating hamburgers will make you a good poet", is falsifiable, but it is not. I would like to know Wolpert's indisputable criteria for distinguishing between a good poet and a bad poet.

Worse still, Wolpert does not appear to understand the problem of induction that Popper tried to solve. Wolpert says, "the theory [Popper's] does not even resolve the problem of induction, for one wants to have a sufficient number of experimental falsifications to be persuaded". The number of "experimental falsifications" that Wolpert considers "sufficient" for him to be "persuaded" is a matter for his own personal psychology and has no logical bearing on the validity of Popper's theory. This is the very problem Hume identified with inductive reasoning and Wolpert has presented a fine example with his silly argument.

**S. C. Francis**

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SIR — When judging the success of Karl Popper, we should perhaps view him as a moralist who prescribes the proper, most productive behaviour of scientists, the path he would have us follow. The philosophers following Kuhn are then viewed as historians, who report how people called scientists actually behave; and Popper's success is best measured by

the number of scientists he has led down his path.

**Robert Eisenberg**

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SIR — John Polkinghorne (*Nature* 360, 378; 1992) berates Lewis Wolpert for remarking, in *The Unnatural Nature of Science*, that "[s]cientific knowledge. ... provides our best way of understanding the world". From the context it is clear that Wolpert means a *rigorous* understanding. Other disciplines such as history or philosophy provide some understanding, but at a different level of precision. Polkinghorne writes that "a painting is more than a collection of specks of paint", implying that science cannot account for the impact of a great painting on the viewer. Nor can it, nor has anybody claimed that it can: perhaps it never will, we cannot know.

Polkinghorne goes on to attack Wolpert for not paying attention "to the source of our intuition of the value of human individuals". Some of us have that intuition, some of us do not. But intuition is extremely fallible and whenever it has been compared with a more scientific approach it has been shown to be less successful, as for example in medical diagnosis. Scientists have intuitions about science, others have intuitions about the existence of God, and Hitler had the intuition that the Aryan race was so superior to others that they should be destroyed.

As Wolpert makes abundantly clear, the difference between scientific intuition and other kinds is that only the former can be tested. The question Polkinghorne poses — how morality originates — is not, as he suggests, necessarily unanswerable by scientists: sociobiologists have already taken a rather crude crack at it. The more important question, which Polkinghorne neither raises nor answers, is how specific ethical or religious beliefs can be defended. In the last analysis they cannot, except by announcing, "I feel it: I have faith." Pace Polkinghorne, Wolpert is surely right in claiming that science is incompatible with religion. Perhaps Polkinghorne should ponder the dilemma that would arise if science were ever able to explain the origins of religious and ethical beliefs: the distinction between explaining and explaining away is a fine one.

**Stuart Sutherland**

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# Research benefits

SIR — Your attempt to correct President George Bush's attempt to cite unexpected benefits of basic research as justification for its funding (*Nature* 358, 530; 1992) is flawed, albeit to a lesser degree than his.

At the time under discussion, I was head of research and development at Cobe Laboratories of Lakewood, Colorado, and also a member of the board of directors of Galen Laboratories. To say that "Cobe modified his invention so that it could be manufactured" is to misunderstand the essence of product development. An "invention", purporting to be a product, that cannot be manufactured is a contradiction in terms. To be an invention, by definition it must be useful. My colleagues and I developed a superb manufacturing process and associated product design (more often than not the two are symbiotic) which continues to this day (some 18 years later) producing high quality medical devices. In so doing we were awarded patents exceeding in number those awarded to Mr Finley Markley.

Our dialyser was initially marketed at \$27.50; later pricing (actual high volume sales, not list) was in the \$16 range. The Cobe technology was not sold to a Yugoslav company. Your statement to the contrary notwithstanding, it is not difficult to measure the impact of Markley's work on those with chronic kidney disease. There were at the time of the introduction of the Medical Inc. dialyser a number of acceptable artificial kidneys. Had Markley's invention not occurred, these other products would have readily supported the patient requirements. Even though the same is true of the Cobe dialyser, my colleagues and I remain proud of the number of patients who received safe and effective treatment from our product.

Your article ignores or even minimizes a far more critical issue. What in the world was the 'management' of Argonne Laboratories thinking of when it allowed tax dollars to be frittered away on a matter that did not relate to its missions and regarding which its organization had no expertise? In short, even if Bush's story had been true, it would not have been cause for celebration but should have raised these questions.

It is the repetition of this story — the arrogant ignoring of the funded mission with the concomitant diversion of funds from the mission to "targets of opportunity" — that is causing the loss of support for the national laboratories on the part of both legislators and taxpayers.

**Donn D. Lobdell**

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# Why were Scud casualties so low?

Steve Fetter, George N. Lewis and Lisbeth Gronlund

**Patriot missiles were returned to the Gulf last week. But they were not the reason for the unexpectedly low casualty rate when Saddam attacked Israel with Scud missiles in 1991.**

IRAQ fired more than 80 modified Scud missiles at Israel and Saudi Arabia during the Persian Gulf War in 1991. These attacks caused 31 deaths, numerous injuries and substantial property damage. With the exception of the Scud that hit a barracks in Dhahran, Saudi Arabia, and killed 28 US soldiers, however, the number of deaths and serious injuries caused by each missile appear to be much lower than one would have expected based on the results of previous ballistic missile attacks. The relatively low casualty rate has been cited by several analysts as evidence of the success of the Patriot missile defence system<sup>1-5</sup>. Others have argued that the same casualty data suggest that the Patriot may not have been very successful<sup>6</sup>.

What effect did Patriot have on casualties during the Gulf War? Here we show that the publicly available data do not support claims that Patriot played a significant role in preventing casualties or damage to buildings in Israel. (We focus on Israel because relatively little data are available from Saudi Arabia.) Why, then, were casualties so low in Israel? To answer this question, we review the effects of previous ballistic missile attacks on cities and discuss various factors that may account for the lower casualty rate in Israel.

## Role of Patriot

The Scud attacks on Israel began on the morning of 18 January 1991, but the first Patriot battery did not become operational until after 11 Scuds had fallen. Of the 38 Scuds that reached Israel, 27 were fired after Patriot was operational; of these, 17 were engaged by Patriot missiles<sup>7</sup>. Most or all of the other ten Iraqi missiles fell in areas not covered by the Patriot batteries deployed near Tel Aviv and Haifa.

According to the US Army's most recently revised estimates of Patriot effectiveness, of the 17 engagements in Israel, only 40% were successful, and the Army had "high confidence" in only 40% of its claimed successful engagements in Israel and Saudi Arabia<sup>8</sup>. Thus, of the seventeen Scuds engaged, at most seven were intercepted, and in only three of these cases does the Army claim high confidence (assuming equal confidence levels in Israel and Saudi Arabia).

The Army claims have been challenged by independent analysts<sup>9,10</sup>. But

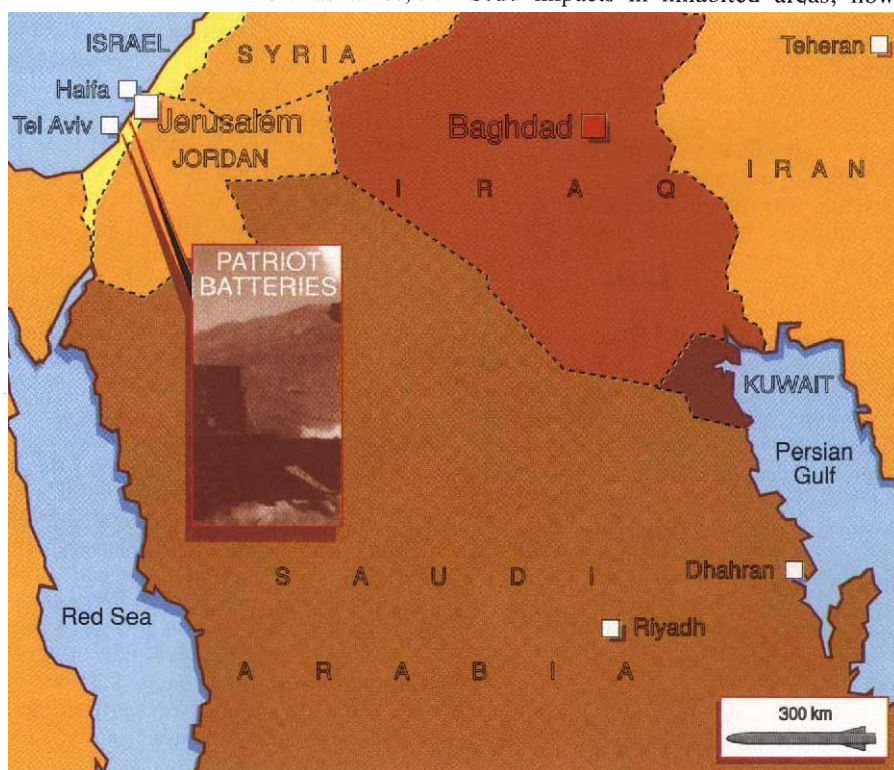
even if we accept the Army's data, Patriot would have reduced the expected casualty rate by no more than 16 to 40%. Moreover, any damage prevented by successful intercepts must be weighed against the damage caused by the four Patriot missiles that struck the ground and exploded in Israel<sup>2,11</sup>, as well as by the debris from intercepted Scuds and from Patriots that detonated above cities. Because of the lack of accurate track data on the Scud and Patriot missiles, the effect of Patriot in preventing casualties and damage in Israel will never be known with any certainty. Given that large statistical fluctuations would be expected in the damage from small numbers of inaccurate ballistic missiles armed with conventional warheads, any effect that Patriot may have had on casualties or damage is lost in the noise.

Casualty and damage statistics before and after Patriot deployment support this conclusion. The 38 modified Scud missiles fired at Israel directly killed two people and injured about 230 more. Almost all the injuries were light, with only ten classified as moderate and one as severe<sup>12,13</sup>. Of the direct casualties, 52

occurred before Patriot was operational (4.7 per missile) and 179 after (6.6 per missile)<sup>12</sup>. All but two of the 179 casualties after Patriot was operational were caused by Scuds that fell in defended areas. Both deaths and most or all of the serious injuries occurred during the period of Patriot defence.

The situation with respect to property damage is more complicated. Theodore Postol has published a translation of a compilation of damage to buildings that appeared in the Tel Aviv newspaper *Ma'ariv*<sup>6</sup>. According to *Ma'ariv*, 2,797 apartments suffered damage before Patriot (254 per missile) and 9,029 after Patriot was operational (334 per missile). Much of the damage to apartments was very light (such as broken windows). If only apartments that were seriously damaged or destroyed are counted, the figures are 40 per missile before Patriot and 34 after Patriot.

Thus, although serious damage to apartments appears to have decreased slightly after Patriot became operational, light damage, injuries and deaths all increased. Given the small number of Scud impacts in inhabited areas, how-



Map of the regions discussed here. Two of the Patriot emplacements defending Israeli targets are shown.

# IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Mary Evans

## Bomb damage by V-2 rocket to brick-row houses in Tottenham, north London, 1944.

ever, these differences are not statistically significant. We can conclude only that the available data do not support the hypothesis that Patriot had a significant effect on casualties or damage.

### Previous ballistic attacks

A review of the effects of previous ballistic missile attacks helps to put the Israeli casualty rate in perspective. Before the 1991 Gulf War, ballistic missiles had been used extensively in war only three times: the Germans launched more than 3,000 V-2 missiles against urban British and European targets during World War II; Iraq and Iran together launched nearly 1,000 missiles against each other's cities during the 1980-88 Persian Gulf War; and the Kabul government fired more than 2,000 Soviet-made Scud missiles against Mujahideen guerrillas in the Afghanistan civil war. In all cases the missiles were armed with conventional high-explosive warheads. Detailed information on casualties and physical damage is available only for the V-2 attacks on London.

**V-2 attacks on London.** From September 1944 until March 1945, Germany launched about 3,000 V-2 ballistic missiles at targets in Britain and continental Europe. Of the approximately 1,400 V-2s fired against Britain, 518 fell in the London civil defence district. In addition, nearly 10,000 V-1 cruise missiles were fired against London. Even though most of the V-1s malfunctioned or were destroyed by British defences, about 2,420 fell on London.

Although the V-2 and the V-1 warheads had nearly equal explosive yields and created roughly equal amounts of property damage per missile<sup>14</sup>, each V-2 impact in London resulted in an average of 4.8 deaths and 11.7 serious injuries, compared to 2.2 deaths and 6.3 serious

injuries per V-1 impact<sup>15</sup>. The lower casualty rate for the V-1 was due primarily to the fact that people in the target area could hear the V-1 approach and could take cover before its warhead exploded, while the V-2 gave no warning of its approach<sup>16</sup>.

**Scud attacks on Teheran.** Only limited information is available on casualties and damage from the Iraqi Scud attacks on Teheran during the so-called "War of the Cities". A total of 189 modified Scud missiles fell on six different Iranian cities from 29 February to 20 April 1988; 135 of these landed in Teheran<sup>17</sup>. On 4 April, after about 125 missiles had fallen, it was reported in Iran that 1,150 people had died and 4,000 had been injured from the missile attacks. Some foreign analysts believe that Iran under-reported casualties to minimize civilian panic, and estimate that 2,000 people died<sup>18</sup>. Thus, about 10 to 15 people were killed and at least 30 injured per missile impact.

If we assume that the circumstances of the Scud attacks on Teheran were similar to those of the V-2 attacks on London, we need only modify the London casualty estimates to account for differences in population density and warhead yield to estimate the expected casualty rate in Teheran. The population densities of Teheran and London before the Scud and V-2 attacks were about 300 and 43 per hectare, respectively; the available information suggests that roughly 20 per cent of the population was evacuated during the attacks in both cases<sup>14</sup>. All else being equal, a given warhead would on average kill and injure about seven times as many people if targeted against Teheran rather than London under the circumstances described here.

The modified Scud reportedly carried

a warhead with 160 to 190 kg high explosive<sup>17</sup>, including the kinetic energy of the missile, which was reduced by its tendency to break up during re-entry, the total energy released on impact would have been equivalent to no more than 250 kg TNT, compared to 900 kg TNT for the V-2 (ref. 14). Because the area subjected to blast damage scales as the yield to the two-thirds power, the lethal area created by the impact of the modified Scud was 0.43 times that of the V-2.

Thus, scaling for differences in population density and warhead yield, the expected casualty rate in Teheran was three times greater than that in London during the Second World War, which can be estimated as 14 deaths and 35 serious injuries per Scud impact. This estimate is in good agreement with the available (albeit sparse and unreliable) information about casualties in Iran cited above. We stress, however, that a detailed comparison with the London experience has not been done because we lack information about many important aspects of the attacks on Teheran (civil defence, construction practices, and so on). As we shall see in the case of the attacks on Israel, such factors can have a significant effect on casualties.

### Scud attacks on Israel

As noted above, the 38 modified Scud missiles fired at Israel resulted in 2 direct deaths, 11 serious injuries, and about 220 minor injuries. The number of Israeli casualties produced by the modified Scud missiles seems remarkably low compared with previous ballistic missile attacks. In fact, the total number of deaths and serious injuries in Israel was less than that caused by just one average missile impact in London or Teheran.

As discussed above, it is necessary to account for differences in population density and warhead yield when estimating casualties. Most of the casualties in the Gulf War occurred in the Tel Aviv metropolitan area, which had a population density of about 70 per hectare in 1991 (ref. 14). As was the case in London and Teheran, many people left Tel Aviv during the missile attacks. Although this evacuation was widely reported in the press, its extent is difficult to estimate, especially as many of the evacuees left only at night, returning to their jobs in the morning. In the absence of better information, we assume the same fraction of evacuees as in London, which gives a night-time population density about 1.6 times that of London during the missile attacks.

The modified Scud missile used against Israel was apparently the same as that used against Iran, and it appears that essentially all the missiles launched by Iraq during the Gulf War also broke



up on re-entry. After applying the appropriate scaling factors for population density and warhead yield to the casualty rates from V-2 attacks on London, one would expect about 3.3 deaths and 8.1 serious injuries per Scud impact in Tel Aviv. Thus, the actual number of deaths that resulted from the 38 missiles launched against Israel was more than 60 times less than what might have been expected based on this straightforward extrapolation from the experience in London. This simple analysis, however, omits several important factors.

**Warning time.** A major difference between the missile attacks on London and Teheran and those on Tel Aviv was that (except in the first two attacks) the Israeli population generally had a few minutes warning time in which to take cover. This was because the United States shared satellite information on Scud launches with Israel. We can account for this very roughly by comparing the casualty rate for the V-1 attacks, for which there was some warning, with that for the V-2 attacks, for which there was no warning. After correcting for its slightly smaller yield, the death rate from V-1 attacks was 0.5 times that from the V-2 attacks (0.58 for serious injuries). Thus, taking into account warning time in addition to population density and warhead yield, the expected rates are reduced to 1.6 deaths and 4.7 serious injuries per missile impact in Tel Aviv.

Another factor that may have reduced casualties is that virtually all the missile attacks on Tel Aviv occurred at night, whereas the attacks on London occurred throughout the day. Thus, most Israelis were at home during the attacks, and on hearing the warnings simply had to go into interior rooms to obtain a degree of shelter.

**Inaccuracy of the modified Scud.** A key factor in limiting the damage to Israel was that most of the modified Scud missiles did not strike populated areas. According to a study of Israeli casualties, only six warhead explosions caused direct casualties<sup>13</sup>.

The basic Scud-B is so inaccurate that, at its maximum range of 300 km, at least half the impacts would fall more than 1 km from the target<sup>19</sup>. Moreover, the pressure the Iraqis were under to launch the missiles quickly, combined with the modifications made to the missile by Iraq to more than double its range, undoubtedly led to a substantial decrease in the accuracy of the missile.

It is difficult to determine the number of Scud impacts in Israeli cities because Israeli censorship regulations did not allow the release of the precise locations of Scud impacts. In the 15 attacks involving a single Scud missile, however, there is little difficulty in identifying the cases in which a warhead detonated in a

populated area: three such warheads exploded in populated areas in or near Tel Aviv; the other 12 warheads presumably fell in uninhabited areas, were duds or were destroyed by Patriot missiles.

It is more difficult to sort out what occurred in the four multiple Scud attacks in which a total of 23 missiles were launched at Tel Aviv and Haifa. The first two attacks occurred before Patriot was operational. Seven missiles were fired at Tel Aviv and Haifa at about 2 a.m. on 18 January 1991, and four missiles were fired at Tel Aviv at about 7 a.m. on the following day. Robert Stein, manager of Advanced Air Defense Programs for Raytheon (Patriot's manufacturer), states<sup>1</sup> that most of these missiles "were not on target, and only very few fell on Tel Aviv". Taken literally, this means that no more than

destroyed by Patriots and, as noted below, at least one of the warheads that landed in Tel Aviv was a dud.

**Blast-resistant dwellings.** One Israeli study of the Scud casualties concluded that the most important factor in reducing casualties was the construction practices used in modern Israeli apartment buildings<sup>13</sup>. The multi-storey apartment buildings erected in Tel Aviv over the past 30 years are constructed with reinforced concrete columns, beams and floors<sup>13,20</sup>. The reinforced concrete elements prevented buildings close to Scud detonations from collapsing and burying their inhabitants. On the other hand, when missiles struck near older, unreinforced masonry buildings, these were often completely demolished, with the roof and walls collapsing.

By contrast, the typical London dwell-

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### **Patriot missile keeping watch over Tel Aviv in January 1991.**

five Scuds were on target; as discussed below, at least one of these did not explode. A Pentagon briefing on 25 January 1991 also implied that no more than five of these missiles landed in populated areas and caused damage<sup>14</sup>.

The other two multi-Scud attacks occurred during the period of Patriot defence: seven missiles were launched on 25 January and five the next day. In the first attack, only two clearly identifiable explosion sites in populated areas were reported: one in a residential neighbourhood and one at a school. The only impact reported in a populated area in the second attack occurred on a deserted beach in Tel Aviv.

Thus, it appears that only about 10 Scud warheads exploded in populated areas in Israel. What happened to the other 28? Some landed well short of Tel Aviv or in the Mediterranean Sea. It is possible that others landed in unpopulated areas in or near Tel Aviv and Haifa but went unreported by the news media. An anonymous Pentagon scientist was reported<sup>7</sup> as saying that Scuds not engaged by Patriot "either fell in the sea or out of range of the Patriot batteries, near towns in the West Bank or in the Negev desert". One study noted<sup>13</sup> that of the missiles launched against Israel, "a number fell in the sea or exploded in the air". Finally, some Scuds may have been

ing was the brick-row house, which often collapsed from the blast of nearby V-2 explosions. The limited data available on the effects of V-2 missiles on reinforced concrete buildings in London indicate that the area of structural damage is roughly eight times smaller than for brick-row houses<sup>16</sup>. Because deaths and serious injuries are due primarily to the structural collapse of buildings, Israeli construction practices were probably a significant factor in reducing the casualty rate relative to that in London.

**Dud warheads.** Some Scud warheads simply failed to detonate. In the second attack on Israel, a warhead penetrated several floors of a building before coming to rest in a ground-floor jewellery shop, where it was recovered intact<sup>21</sup>. There were also reports that one or more of the missiles fired at Israel did not carry explosive warheads<sup>22</sup>. Given the ample evidence that many of the warheads that landed in populated areas did explode, however, the fraction of duds was probably small.

**Civil defence.** Most of the beneficial effect of civil defences has already been accounted for in the discussion of warning time and construction techniques. Israel devoted substantial resources to rapid rescue efforts, and several people were reportedly rescued from collapsed buildings. But it is unlikely that Israeli

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### Scud launched from Iraq hits Tel Aviv at night in January 1991.

efforts were much better than those of the British rescue services, which had experienced several years of German air attacks by the time of the V-1 and V-2 launches. Thus, civil defence probably played a minor role in reducing the casualty rate relative to that in London.

**Coincidence.** Substantial statistical fluctuations are expected in attacks involving only a small number of inaccurate ballistic missiles with small damage radii. Because only about ten warheads detonated in Israeli cities, luck must have played a crucial role in determining the overall casualty rate. Indeed, there is considerable anecdotal evidence that good fortune was important in reducing casualties in Israel. Of the warheads that detonated in Israeli cities, one hit the only empty lot in a densely populated neighbourhood<sup>23</sup>, three others hit a park, a factory and an unfinished shopping mall during the night. Two Scuds landed near unoccupied buildings: an underground bomb shelter at a municipal centre and a school. Even when Scuds severely damaged occupied buildings, casualties were remarkably low: a missile that landed in an alleyway between several apartment buildings and caused one building to collapse killed only one person, and an attack that destroyed a two-storey house and severely damaged

several others also killed only one person; two people reportedly survived only because they disobeyed government instructions and went to their basement bomb shelter.

### Conclusions

Given the number of missiles fired, casualties in Israel at first appear to be very low compared with what could have been expected on the basis of previous ballistic missile attacks. But several characteristics of the modified Scud helped to limit casualties, most notably the inaccuracy of the missile (only about one-quarter of the missiles detonated in populated areas in Israel), together with its small warhead, its break-up on re-entry and an unknown but probably small number of dud warheads.

Several other factors seem to have been important in reducing casualties. The difference in the V-1 and V-2 casualty rates suggests that the warning time provided by US launch-detection satellites reduced casualties by a factor of two. In addition, coalition air attacks forced Iraq to launch missiles after dark, when most Israelis were at home.

Taken together, these factors allow us to estimate, based on the London V-2 experience, that 16 deaths and 47 serious injuries would have been expected from

the roughly 10 Scud detonations in Israeli cities, excluding casualties that may have been caused by the 4 Patriot impacts. Two deaths and eleven serious injuries actually occurred. Much of this apparent discrepancy may be due to Israeli construction practices, which prevented the collapse of heavily damaged buildings. As noted above, the limited data from London indicate that the area of destruction for reinforced concrete buildings was about eight times smaller than that for brick-row houses.

Given the significant statistical fluctuations that would be expected in a casualty rate based on such a small number of explosions, these factors could account for the observed casualty rate in Israel. However, anecdotal evidence also suggests that luck helped to reduce casualties. As illustrated by the Scud that hit the barracks in Dhahran and killed 28 soldiers, shifting the impact point of a single missile by tens of metres could have changed the casualty statistics dramatically, given the small number of warheads that detonated in Israeli cities.

The available evidence does not support claims that the Patriot missile defence system significantly reduced casualties in Israel. While Patriot might have destroyed some warheads that would otherwise have caused casualties, the available data contain no evidence for a net reduction in casualties or damage due to Patriot.

Several important lessons can be drawn from the experience of the Gulf War. The widely held belief that ballistic missiles are themselves weapons of mass destruction is simply incorrect, as demonstrated by this as well as by past episodes. It is the nature of the warhead, not the mode of delivery, that counts. Even attacks with conventional warheads could be far more lethal. If a few missiles had struck crowded buildings, or if the missiles had been more accurate or had carried larger warheads, or if the attacks had taken place during the day rather than during the night, the number of deaths could have been many times greater. Finally, advance warning of missile attack, which in this case was made possible by satellites, can save lives by allowing people to shelter or to take other evasive action. □

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- Stein, R. *Int. Security* **17**, 200 (1992).
- Zraket, C. *Defense News* 31 (9 December 1991).
- Pfaltzgraff, R. L. Jr *Wall Street J.* A20 (8 April 1992).
- Detroit News* 8 (13 April 1992).
- Boot, M. *Christian Sci. Monitor* 9 (23 September 1992).
- Postol, T. A. *Int. Security* **16**, 119–171 (1991–92).
- Schmidt, E. *New York Times* (Int. edn) A8 (31 October 1991).
- Schmidt, E. *New York Times* A11 (8 April 1992).
- US General Accounting Office. *Operation Desert Storm: Data Does Not Exist to Conclusively Say How Well Patriot Performed* (GAO/NSIAD-92-340, September 1992).
- Lewis, G. N. & Postol, T. A. *An Evaluation of the Army Report "Analysis of Video Tapes to Assess Patriot Effectiveness"* (MIT Defense and Arms Control Studies Program, September 1992).
- Kaplan, F. *Boston Globe* 1 (5 May 1991).
- Bleich, A. et al. *J. Am. med. Ass.* **268**, 613–615 (1992).
- Karsenty, E. et al. *Israeli J. med. Sci.* **27**, 603–607 (1991).
- Lewis, G. N., Fetter, S. & Gronlund, L. *Casualties and Damage from Scud Attacks in the 1991 Gulf War* (MIT Technology, Defense and Arms Control Studies, January 1993).
- Dunn, C. L. *The Emergency Medical Services* **1**, 174; 179 (HMSO, London, 1952).
- US Strategic Bombing Survey, *Physical Damage Division V-Weapons in London Report No. 152* (January 1947).
- Carus, W. S. & Bermudez, J. S. Jr *Jane's Soviet Intelligence Review* **2**, 242–248 (1990).
- Bierman, J. *Maclean's* 34–36 (18 April 1988).
- Zaloga, S. *Int. Defense Rev.* **11**, 1427 (1988).
- Rudge, D. *Jerusalem Post* 8 (22 February 1991).
- Brinkley, J. *New York Times* 1 (20 January 1991).
- Flight Int.* 13 (13–19 March 1991).
- Chartrand, S. *New York Times* A7 (19 January 1991).



# New horizons in inner space

**The voids inside fullerene molecules and their larger relatives offer a cosseted environment in which unusual physics and chemistry may be possible. Fundamental science, as well as applied research, should benefit.**

THE old riddle has it that there are two sides to a sphere — inside and outside. From the outside, the closed carbon cages of fullerenes and 'nanotubes' are potential building blocks for chemistry and technology. From this perspective,  $C_{60}$  itself can be regarded as a kind of giant pseudo-atom. But from the inside, these carbon structures acquire a new character: that of a microcosm (or perhaps nanocosm).

The interior phase of fullerenes such as  $C_{60}$ , the realm of so-called endohedral chemistry, has been widely discussed. Formation of the hollow carbon clusters by laser ablation of a composite of graphite and lanthanum oxide creates fullerene-lanthanum complexes in which the metal atoms appear to be inside the fullerene cage. The stability of these complexes in solvents argues strongly that the metals are encapsulated as endohedral species.

But there is not much room inside the fullerenes known at present —  $C_{82}$  has an inside diameter of about five ångströms, enough to accommodate just three or four metal atoms. Sumio Iijima's discovery of carbon nanotubes in 1991 led Richard Smalley to predict potentially more interesting ways of exploiting their interior phase: for example, by trapping metal atoms "like peas in a pod". The tubes might thereby become 'nanowires'.

But how to get the atoms inside? As formed in the now-standard arc-discharge technique, the carbon tubes are capped at each end, presumably as a result of pentagonal defects in the hexagonal graphite-like sheets. These cause the sheets to curl up and close upon themselves, avoiding dangling bonds. These relatively high-energy caps are the most promising target for attempts to make an opening.

That seems to be borne out by experiments of Iijima and P. M. Ajayan reported on page 333 of this issue. Among samples of closed nanotubes, they deposit microscopic particles of lead from a laser-evaporated vapour. On annealing in air at 400 °C (above lead's melting point), the caps are destroyed and molten material is sucked inside the tubes by capillary action. The mechanism of cap destruction is not clear, although both the metal particles and air seem to be essential — some kind of metal-catalysed oxidation is a possibility.

Whether the material that fills the tube is pure lead or a compound is also unclear, although apparently it is not a simple oxide. Moreover, the filling is mostly amorphous, and so it may not be the ideal metallic nanowire that might have been hoped for. But it will be interesting to see what conductivity measurements reveal. Transmission electron microscopy shows some partially

contact angles remain well defined in the filled nanotubes, for instance), the character of phase transitions shows a pronounced dependence on the size and the dimensionality of the system. In cylindrical pores, correlation lengths can diverge only along the pore axis, so phase transitions should be pseudo-one-dimensional, quite unlike those in three-dimensional bulk materials. Liquid-vapour transitions (capillary condensation) and superfluid transitions in porous media have been studied in the past, but open, uniform cylindrical nanotubes might provide the ideal pores much needed in these investigations.

Solid-liquid transitions in nanometre-scale pores (capillary melting/freezing) have been investigated only theoretically (G. Navascués & P. Tarazona, *Mol. Phys.* **62**, 497; 1987), and it is here in particular that studies of filled nanotubes might bear fruit, as the change of phase in a single tube may be visible with electron microscopy. As with capillary condensation, the expectation is that the freezing or melting transition will be shifted in a narrow pore relative to the bulk; but in addition, constraints on packing in the interior phase may generate a rich subtlety in the phase diagram, and there is the intriguing possibility of a solid-liquid critical point, at which the two phases lose their distinct identity. Symmetry arguments appear to rule out this possibility for bulk materials.

Another potential approach to the generation of filled carbon nanostructures is to create them with the filling *in situ*, in the same way that endohedral fullerene complexes are formed. That is the very trick that has enabled Rodney Ruoff and colleagues (*Science* **259**, 346-347; 1993) and Yahachi Saito and co-workers (*Jap. J. appl. Phys.*, in the press) to create closed, concentric polyhedral carbon shells (like short, fat nanotubes) containing what appears to be crystalline lanthanum carbide ( $LaC_2$ ). The encapsulated material (which is normally hygroscopic) is protected against several days' exposure to air. Here, then, is a new kind of airtight wrapping for readily degraded substances. Based on experience with synthetic, macrocyclic cages such as the hemicarcerands of Donald Cram, it may be that these structures will provide a protected environment for all manner of delicate chemistry.

**Philip Ball**

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Lanthanum carbide crystal inside a 30-nm carbon polyhedron. (Courtesy Y. Saito.)

crystalline regions of filling. Perhaps this crystallinity will be improved if a way can be found to introduce the material more slowly into the tube — by controlling the wetting dynamics.

What applications this new nanocomposite might offer will not be apparent until its electronic and mechanical properties have been measured. The empty tubes are themselves predicted to have immense tensile strength along the axis and to be semiconducting or metallic, depending on the detailed structure of the cylindrical sheets.

Some of the most immediate benefits, however, may be for fundamental research. In particular, the opening and filling of nanotubes should cast new light on how matter behaves under confinement. Previous studies of this sort, aimed for example at understanding the effect of confinement on phase transitions, have relied on porous media such as Vycor silica glass, which can soak up simple liquids and gases. But the non-uniform pore sizes and shapes of these materials complicates interpretation of the results.

Although in many respects concepts relating to bulk matter remain surprisingly well suited to describing systems of nanometre dimensions (menisci and

# Genes tell a new whale tale

Michael Novacek

THE grand array of living whales (mammalian order Cetacea) is customarily organized into two groups: the suborder Odontoceti, the toothed forms, including the sperm whales and diverse dolphins and porpoises; and the suborder Mysticeti, the baleen whales, including that most leviathan of animals the 100-tonne blue whale. The widely accepted odontocete–mysticete split is, however, contradicted in new findings of Milinkovitch *et al.* on page 346 of this issue<sup>1</sup>. Data from DNA sequences of 12S and 16S mitochondrial ribosomal RNA genes indicate a closer relationship between the sperm whales and the baleen whales than between the sperm whales and other alleged odontocetes. This suggests that the remarkable echolocating behaviour believed to occur in all toothed whales (including the sperm whales) was either secondarily lost in baleen whales or was gained convergently in different odontocetes.

The mitochondrial DNA study is intriguing because it suggests that odontocetes are not monophyletic, that is, they do not comprise a distinct assemblage with an exclusive common ancestor. Rather, the data imply that odontocetes are, in the current systematic parlance, paraphyletic — their nearest common ancestor was also shared with mysticetes. It is noteworthy that this contradiction is part of an overall pattern that otherwise matches well with standard morphological results. The mitochondrial data clearly support the monophyly of such respected groupings as beaked whales (Ziphioides), rorqual baleen whales (family Balaenopteridae), sperm whales (Physeteroidea), dolphins

(Delphinidae) and porpoises (Phocoenidae). The sperm whale/baleen whale grouping thus represents a major anomaly in a case where morphology and molecular data otherwise agree.

Milinkovitch *et al.* anticipate scrutiny of these provocative results by applying several techniques for tree construction and evaluation that reflect various schools of systematics. All approaches yield results that basically converge on the pattern derived from cladistic parsimony. The familiar cetacean groups, as well as the unexpected sperm whale/baleen whale connection, are maintained under virtually all circumstances. The authors do not describe the stability of the latter grouping among trees of all possible lengths<sup>2</sup>. They do, however, note that constraining the data to support the monophyly of odontocetes produces a tree at least eight steps longer than the shortest trees based on unweighted parsimony analysis. A similar, though less taxonomically enriched, result is derived from analyses of previously published data on myoglobin amino acid sequences<sup>3</sup>.

The association of sperm and baleen whales has obvious evolutionary implica-

tions. It has been assumed that the remarkable echolocating behaviour of toothed whales was not present in the ancestral cetacean. Under this view, mysticetes simply retain a hearing system and related behaviour closer to the ancestral condition. The branching suggested by ribosomal DNA sequences,

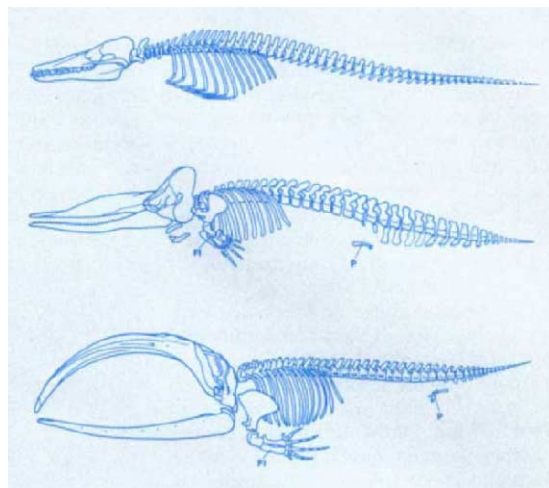


FIG. 2 Three whale groups. Top, A fossil archaeocete (*Dorudon osiris*); middle, an odontocete, the sperm whale (*Physeter macrocephalus*); bottom, a mysticete, the bowhead whale (*Balaena mysticetus*). Fl, flipper; P, pelvic girdle with rudimentary hind limb. From ref. 11.

however, allows that echolocation might have been secondarily lost in baleen whales. Alternatively, cetacean echolocation may have evolved at least twice; once within the sperm whale/baleen whale clade and once within the odontocetes outside this clade.

The new arrangement is also applied to the question of divergence times for these whales. On the basis of rates assumed for transversion changes in ribosomal mitochondrial genes of ungulate mammals, Milinkovitch *et al.* postulate a 10–13-million-year age for the split between sperm whales and baleen whales. The authors note that such an estimate is not contradicted by fossil evidence, but this is incorrect. Earliest members of the baleen-whale family, the Balaenidae, as well as sperm whales, are found in 23-Myr-old strata in South America<sup>4</sup>. Furthermore, archaic toothed mysticetes have now been identified from early Oligocene (35 Myr old) beds<sup>5</sup>. Thus, estimates of age of divergence based on gene rates are clearly in conflict with the known fossil record.

Molecular studies are often judged harshly for not drawing on a diverse sampling of taxa. In this case, the mitochondrial DNA sequences reflect reasonable representation — information on all the cetacean families derives from 16 species of whales, dolphins and porpoises. Outgroup analysis is, how-

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# Making the right connections

Haig Keshishian

ever, restricted to cow, ass, human and sloth. Despite the agreement of this study with morphological evidence for a close artiodactyl/cetacean relationship (reviewed in ref. 6); broader issues of cetacean phylogeny are thus somewhat out of the scope of these ribosomal data. Another limitation resides in the simple fact that the third major group of whales (Fig. 2), the archaeocetes, is represented exclusively by fossils. The problem is relevant because some alleged archaeocetes associate more closely with mysticetes than they do with each other<sup>4</sup>. Both morphological<sup>7</sup> and DNA-sequence data<sup>8</sup> from fossils have critically changed branching relationships based solely on living representatives. This invites expanded analyses of archaeocete relationships with other whale groups.

The results also compel us to look at the original premise they challenge. A mysticete-odontocete divergence is correctly characterized by Milinkovitch *et al.* as "traditional", but is it firmly established? Few morphological studies explicitly address this problem. One recent cladistic study identified several cranial traits that support the monophyletic grouping of odontocetes<sup>9</sup>. Nonetheless, some of the features recruited for this grouping are questionable (the asymmetry of cranial bones is not found in some odontocetes<sup>4</sup>) and most other features seem closely correlated as components of the echolocating system. If the morphological basis for the mysticete-odontocete split is at least open to further scrutiny (see also ref. 10), the mitochondrial results ironically seem less provocative. Even so, the molecular data are enlightening — they suggest that the impressive morphological database on living and fossil whales might benefit from analyses reflecting a more expansive view of alternatives for whale phylogeny. □

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GENETIC approaches have been used to study the development of neural connections in organisms as diverse as nematodes, zebrafish and mice. It is therefore ironic that for the classic genetic workhorse — the fruit fly *Drosophila melanogaster* — relatively little has been reported along these lines. But as several studies over the past few years show, the *Drosophila* embryo is indeed well suited for the genetic analysis of synaptic development. In the most recent, reported on page 350 of this issue<sup>1</sup>, Broadie and Bate find that the way in which motor neurons seem to influence the expression and localization of neurotransmitter receptors on muscle cells in *Drosophila* is intriguingly reminiscent of the situation in vertebrates.

Many insect embryos and larvae have highly stereotyped arrays of muscle fibres. These were first described in 1760 by the Dutch naturalist Pierre Lyonnet, who illustrated the innervation of larval moth muscles in a collection of magnificent copperplates<sup>2</sup>. In *Drosophila*, each side of an abdominal segment contains an array of 30 muscle fibres, with the cells defined by their location, and in some cases by characteristic molecular expression patterns. The musculature is innervated by about three dozen glutamatergic motor neurons<sup>3</sup>, a subset of which coexpress other neurotransmitters, including neuropeptides and monoamines.

The key events in synaptogenesis begin when the embryo has completed about half its development (that is, 13 hours at 25 °C), and after the muscle fibres have formed<sup>3-5</sup>. In less than 4 hours the motor-neuron growth cones have differentiated into miniature synaptic endings, each anatomically prefiguring the mature contacts of the larva. These events have been studied at the single-cell level in the RP motor neurons, whose readily accessible cell bodies lie clustered on either side of the dorsal midline<sup>3,4</sup>. At least four RP axons follow a common path in the central nervous system, diverging in the periphery to make specific connections with their target muscle fibres. Initially the growth cones send filopodial processes widely to contact multiple muscle fibres. At their preferred targets the endings promptly differentiate, withdrawing processes that are in contact with adjacent fibres<sup>3-5</sup>. The accuracy of synaptic wiring in *Drosophila* is probably mediated by direct cellular recognition of the muscle fibres by the growth cones. This theory has been tested using both microsurgical and genetic methods to

challenge RP motor neurons with altered numbers and patterns of muscle targets. As in chick and zebrafish embryos, *Drosophila* motor neurons have intrinsic preferences for specific muscles<sup>6,7</sup> as synaptic partners.

What are the molecular mechanisms governing synaptic recognition in *Drosophila*? More than a decade ago several genes were identified that are needed to assemble the neural circuit involved in the adult light-off jump reflex<sup>8</sup>. A similar approach, to detect defects in embryonic neuromuscular connections, holds great promise. Nevertheless, using immunocytochemistry and enhancer-detector methods, an intriguing pattern of membrane and cell-surface glycoproteins has been found on embryonic motor-neuron growth cones and muscle fibres. For example fasciclin III, a member of the immunoglobulin superfamily, is coexpressed by motor neuron RP3 and its target muscle fibres at the site where the synapse forms<sup>1,4</sup>. Two other membrane glycoproteins, connectin and Toll, are expressed transiently at developing synapses<sup>9</sup>, and all three can mediate cell adhesion when expressed in cultured cells. The expression of both fasciclin III and connectin occurs independently of cues from the innervating axons<sup>1</sup>. However, homozygous *FasIII* embryos, which do not express fasciclin III, have essentially normal synaptic wiring, suggesting that any role of fasciclin III in connectivity must involve interaction with other molecules, possibly in a combinatorial fashion.

The functional development of the embryonic synapses can be followed in cultured fillets, made by flattening a dorsally incised embryo against a glass slide. Broadie and Bate<sup>5</sup> have used whole-cell voltage clamping to examine the embryonic development of both glutamate receptors and ion channels. As well as detecting muscle-fibre responses caused by the motor-neuron transmitter, they used glutamate iontophoresis to identify the location and number of glutamate receptors, by monitoring individual receptor openings. During the early stages of normal innervation the muscle fibre expresses as few as 25 large-conductance glutamate receptors. As innervation progresses, the receptors localize to the site where the synapse forms, and in later stages a second wave of glutamate receptor expression follows. Whether synaptic activity has a role in the refinement of synaptic contacts in the embryo, a feature of vertebrate neuromuscular development, has yet to be determined in

1. Milinkovitch, M. C., Ortí, G. & Meyer, A. *Nature* **361**, 346–348 (1993).
2. Hillis, D. M. in *Phylogenetic Analysis of DNA Sequences* (eds Miyamoto, M. M. & Cracraft, J.) 278–294 (Oxford University Press, 1991).
3. Czelusniak, J. *et al.* in *Current Mammalogy* (ed. Genoways, H. H.) 545–572 (Plenum, New York, 1990).
4. Barnes, L. G. in *Mammals: Notes for a Short Course Organized by P. D. Gingerich and C. E. Badgley* (ed. Broadhead, T. W.) 139–154 (University of Tennessee Department of Geological Sciences Studies in Geology, 1984).
5. Fordyce, R. E. *N.Z. J. Geophys.* **32**, 395–400 (1989).
6. Novacek, M. J. *Nature* **356**, 121–125 (1992).
7. Gauthier, J., Kluge, A. G. & Rowe, T. *Cladistics* **4**, 105–209 (1988).
8. DeSalle, R., Gatesy, J., Wheeler, W. & Grimaldi, D. *Science* **257**, 1933–1936 (1992).
9. Heyning, J. E. *Contributions in Science, Natural History Museum of Los Angeles County* **405**, 1–64 (1989).
10. McKenna, M. C. in *Molecules and Morphology in Evolution: Conflict or Compromise?* (ed. Patterson, C.) 55–93 (Cambridge University Press, 1987).
11. Oelschläger, H. A. *Natur Mus. Frankf.* **108**, 317–333 (1978).

*Drosophila*. There is nevertheless good evidence that neuromuscular activity influences the morphogenesis of body-wall synapses, at least when assayed in the larva. Where electrical excitability is increased using potassium channel mutants, there is a corresponding elaboration of the size and complexity of the motor endings<sup>10</sup>.

The new paper by Broadie and Bate<sup>1</sup> looks at the effects of total body-wall denervation on the muscle cells. Embryos mutant for the *prospero* gene have extensive errors in the CNS, with many efferent neurons failing to exit the CNS. The *prospero* product is localized to the nuclei of neural precursor cells, and is involved in establishing the appropriate cell lineages. The mutation was used to separate genetically those events in synaptogenesis that are induced by motor neurons from those that occur intrinsically in the musculature. It turns out that innervation plays no part in the normal accumulation of either fasciclin III or connectin to synaptic sites, or in the initial expression of glutamate receptors. Nevertheless, the localization of the receptors to the predicted synaptic sites did not occur in the absence of the innervating motor neuron, and the second wave of receptor expression which normally follows innervation was also disrupted in the denervated *prospero* muscle fibres. When the few developing motor neurons in *prospero* mutants occasionally established connections on ectopic sites, receptors accumulated at those locations. In *Drosophila*, glutamate receptors accumulate at ectopic innervation sites even when native motor neurons have made functional connections at their correct locations, as is seen for ectopic neuromuscular synapses in the *bithorax* mutant<sup>11</sup>.

Events involved in the expression and localization of *Drosophila* neurotransmitter receptors are reminiscent of those occurring at vertebrate neuromuscular junctions, raising the possibility that common mechanisms may be at work. Many questions are raised by the observations made in the fruit fly. Is the

receptor expression and localization activity dependent? Does the motor neuron release receptor-inducing activities, resembling the vertebrate ARIAS? Are there molecules involved that organize receptors, similar to vertebrate agrin? In a system using multiple neurotransmitters, how do the appropriate receptors associate with the correct presynaptic processes? *Drosophila*, with

its beautifully patterned array of body-wall synapses is just the organism for tackling these and other questions, where hypotheses about development can often be phrased in terms of single neurons and single genes. □

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## CLIMATE CHANGE

# The elusive Arctic warming

John E. Walsh

TEMPERATURE data obtained from drifting ice stations and aircraft over the Arctic Ocean provide no evidence of surface warming in that region over the past four decades. This finding, reported by Kahl *et al.* on page 335 of this issue<sup>1</sup>, apparently runs counter to the grain of the greenhouse-induced climate changes projected by global climate models. Experiments with climate models have indicated that the greenhouse warming will be amplified in the polar regions, at least during the winter half of the year<sup>2</sup>. The new results are all the more striking because they indicate a statistically significant decrease in surface temperatures in the western Arctic Ocean (70–80° N, 90–180° W) during winter and autumn of the 1950–90 period. Interestingly, the winter temperatures at 1–1.5 km altitude have apparently warmed by 3.4 °C over 40 years, while the surface has cooled by 4.4 °C, thereby increasing the strength of the temperature inversion over this range by a striking 7.8 °C.

When viewed in a broader context, however, these findings (apart from the inversion) are not necessarily at odds with climate-model projections. The drifting ice stations, from which were launched the balloon-borne radiosondes that provided all of the authors' temperature profiles for 1962–90, have to be sited on the thick pack ice of the central Arctic Ocean. So they do not sample the potentially sensitive marginal ice zone, where the ice retreat generally results in the greatest warming in CO<sub>2</sub>-doubling experiments of climate models<sup>3</sup>.

The broad latitudinal range of many model-derived Arctic warming patterns is attributable to the seasonal migration of the boundary of a reduced ice cover: a model's newly created open water typically extends a few degrees north of the present ice edge in winter, but it extends well into the central Arctic Ocean (or even to the pole) during summer. The distribution of Russian drifting station tracks in Kahl *et al.*'s Fig. 1 (see page 335) indicates that only one track ex-

tends as far south as 75° N in the Western Arctic domain, and one other track extends as far south as 75° N in Fram Strait. Many of the dropsonde profiles were obtained from the climatologically warmer marginal ice zone of the western Arctic, but all these measurements were made in the first 12 years of the 40-year study period.

The contrast between the central Arctic Ocean and the subarctic assumes greater significance when one examines the temperature trends derived from other data sources<sup>4</sup>: land-station and ship reports. From such data, we have found<sup>5</sup> a high-latitude warming over the past 30 years, but primarily over the northern land areas<sup>5</sup> (see figure). The few areas of noteworthy oceanic warming (north of Svalbard, Kara-Laptev Seas) are south of the ice-station and dropsonde data. Interestingly, according to the 1992 Supplementary Report of the Intergovernmental Panel on Climate Change<sup>6</sup>, CO<sub>2</sub>-doubling experiments with some of the more advanced coupled atmosphere-ocean climate models (UK Meteorological Office, Geophysical Fluid Dynamics Laboratory, Princeton) project a pattern similar to that emerging from all the data: strongest warming over land, weaker warming over the subpolar oceans, and little warming (or even a cooling) over the climatically delicate North Atlantic Ocean near southern Greenland. As is the case in the recent (1961–90) data, the projected warming over land is stronger in winter than in summer. Indeed, the summer trends must be negligible over ice-covered oceans, where the summer surface temperatures are kept at 0 °C by melting snow and ice. The land-sea contrast of the recent warming is consistent with a forced variation of climate.

Data-based indications of high-latitude warming are not limited to the past few decades. Tree-ring chronologies from high-latitude sites have been used to reconstruct annual temperatures for an "Arctic zone" and for the Northern Hemisphere<sup>7</sup>. The low-frequency varia-

1. Broadie, K. S. & Bate, M. *Nature* **361**, 350–353 (1993).
2. Lyonnet, P. *Traité anatomique de la chenille qui ronge le bois de Saule* 2nd edn (De Hondt, Hove, Amsterdam, and London, 1762).
3. Sink, H. & Whittington, P. M. *J. Neurobiol.* **22**, 298–311 (1991).
4. Halpern, M. E., Chiba, A., Johansen, J. & Keshishian, H. *J. Neurosci.* **11**, 3227–3238 (1991).
5. Broadie, K. S. & Bate, M. *J. Neurosci.* (in the press).
6. Sink, H. & Whittington, P. M. *Development* **113**, 701–707 (1991).
7. Chiba, A., Hing, H., Cash, S. & Keshishian, H. *J. Neurosci.* (in the press).
8. Thomas, J. B. & Wyman, R. J. *Nature* **298**, 650–651 (1982).
9. Nose, A., Mahajan, V. B. & Goodman, C. S. *Cell* **70**, 553–567 (1992).
10. Budnik, V., Zhong, Y. & Wu, C.-F. *J. Neurosci.* **10**, 3754–3768 (1990).
11. Cash, S., Chiba, A. & Keshishian, H. *J. Neurosci.* **12**, 2051–2064 (1992).



## RÉSUMÉ

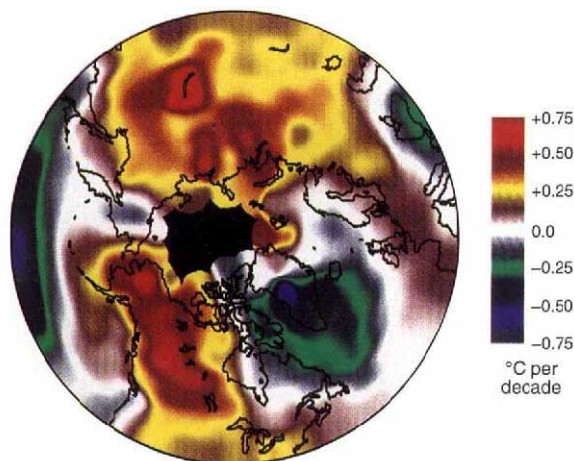
tions are similar in the Arctic and hemisphere time series, and the Arctic variations generally appear as amplifications of the latter. Interestingly, the Arctic temperatures deduced from the tree rings show a century-scale warming interrupted by a cooling from the 1940s to approximately 1970; the instrumental temperatures indicate that the warming over the high-latitude land areas was most pronounced after the late 1960s.

A highly relevant model experiment has recently been performed by Oglesby and Saltzman<sup>8</sup> as a part of MECCA (Model Evaluation Consortium for Climate Assessment). A short-term climate simulation using CO<sub>2</sub> concentrations that increased from 330 to 342 parts per million (corresponding approximately to the increase from 1973 to 1983) showed no significant warming in the Arctic, even in winter. However, when the model was run with CO<sub>2</sub> prescribed to increase from 330 to 450 parts per million (the change projected for 2048), significant warming occurred in the Arctic. The implication is that natural variability will overwhelm the greenhouse signal in the Arctic over a ten-year period, but that the Arctic warming is detectable as a statistically significant signal in a model simulation of 70–100 years.

These studies indicate that climate-model results are not necessarily at odds with recent temperature variations in high latitudes. One may also point to compatibilities between precipitation data of recent decades and model projections of an increase of high-latitude precipitation as greenhouse-gas concentrations increase. Wetter conditions in high latitudes are quite plausible if the greenhouse scenario includes surface warming, higher evaporation rates, and a greater moisture-holding capacity of the warmer atmosphere. Recent data analyses have shown an increase of precipitation in the northern extratropics during the twentieth century<sup>9,10</sup>, and the increase over northern Canada and Alaska during the past few decades has been especially large<sup>11</sup>.

One may conclude that the greenhouse projections of climate models are consistent, in several respects, with high-latitude data from the past few decades. However, the model projections are not consistent with other observational findings, such as the remarkable strengthening of the wintertime temperature inver-

sions reported by Kahl *et al.* from over the Arctic Ocean. Subtleties in the model formulations of Arctic boundary-layer processes (including cloud/radiative interactions) may well be at the root of this discrepancy. Certainly, the vertical resolution of current models is inadequate. Alternatively, the changing spatial and temporal distributions of the temperature data relative to the climato-



Trends of annual temperature (°C per decade) for 1961–90 based on land-station and marine-ship reports. Data from the Arctic Ocean are not included. The analysis was obtained by spatially filtering local trends using a 400-km radius of influence. All points in black area at centre are more than 400 km from locations for which data were consistently available. (From ref. 5.)

logical spatial pattern may contribute to the apparent discrepancy. As the breakdown of the near-surface temperature inversion contributes substantially to the polar amplification of the greenhouse warming projected by models, the resolution of this discrepancy deserves high priority. One possible approach is to replicate Kahl *et al.*'s sampling strategy, both spatially and temporally, using output from a climate model run with the changing CO<sub>2</sub> concentrations of the period 1950–90. □

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1. Kahl, J. D. *et al.* *Nature* **361**, 335–337 (1993).
2. Houghton, J. T. (ed.) *Climate Change: The IPCC Scientific Assessment* (Cambridge University Press, 1990).
3. Maxwell, J. D. & Barrie, L. B. *Ambio* **18**, 42–49 (1989).
4. Jones, P. D. *et al.* *J. Clim. appl. Met.* **25**, 161–179 (1986).
5. Chapman, W. L. & Walsh, J. E. *Bull. Am. met. Soc.* **74**, 3–15 (1993).
6. Gates, W. L. *et al.* in *Climate Change 1992: The Supplementary Report to the IPCC Scientific Assessment* (eds Houghton, J. T. *et al.*) 103–134 (Cambridge University Press, 1992).
7. Jacoby, G. C., Jr & D'Arrigo, R. *Climatic Change* **14**, 39–59 (1989).
8. Model Evaluation Consortium for Climate Assessment, *MECCA Progress and Status* (MECCA Communications Office, Brookfield, Wisconsin, 1992).
9. Diaz, H. F. *et al.* *J. geophys. Res.* **94**, 1195–1240 (1989).
10. Vinnikov, K. Y. *et al.* *J. Clim.* **3**, 662–677 (1990).
11. Karl, T. R. *et al.* *J. Clim.* (in the press).

## Fading vision

The reflected glory of the supernova SN1987A is on the wane, according to A.P.S. Crotts and W.E. Kunkel (*IAU Circ.* No. 5691; 1993). When the precursor star exploded in the Large Magellanic Cloud 180,000 years ago, it launched an expanding shell of intense light into space. The first astronomers knew of it was when that shell reached Earth in February 1987. But within a year, light echoes from material around the supernova appeared — a ring of gas was illuminated first, then a bipolar nebula of dust and gas either side of the star (ejected during its dying phase). The light reflected by dust has now faded from view, so that the part of the shell receding from us must now have reached empty space. The nebula must extend 2.2 light years beyond SN1987A (our nearest star is 4.2 light years away), Crotts and Kunkel say. The gas, heated and ionized by the flash, will continue to glow for some time.

## Big blows

The gales that lashed Britain in 1987 and 1990 have blown some good: they have helped J. R. L. Allen estimate characteristics of winds that felled trees long ago (*Phil. Trans. R. Soc.* **B338**, 335–364; 1992). Until now, estimates of wind direction in the geological past have largely relied on deposits such as dune fields, laid down in arid conditions. The abundant data from trees felled in the recent storms have allowed Allen to relate wind direction and strength to the manner in which trees are felled (bent, snapped or uprooted). Allen applied the findings to ancient trees blown over between 6,000 and 2,500 years ago. It turns out that Britain's weather, at least in the area of southwest England where Allen collected his data, seems to have been as bad then as it is now.

## Take five

Tests for an exotic fifth force, which would look like a composition-dependent correction to gravity, continue to occupy a small band of dedicated physicists. Having ruled out any compositional variation in gravitational acceleration on a terrestrial scale, the group of E. G. Adelberger has turned its attention to cosmological dark matter (G. Smith *et al.* **70**, 123–126; 1993). If different varieties of ordinary nuclear matter can be suspected of generating a fifth force, what of the invisible component, plausibly exotic, that astronomers say makes up 90% of our Galaxy? In the event Smith *et al.* find that galactic dark matter has no differential effect on aluminium, beryllium or copper (their test masses of choice), which would have shown up as a diurnal signal from their apparatus as the Earth rotated. Nor does any effect show up from extragalactic material. Where to look now?

# GABA receptors, granule cells and genes

Mark Farrant and Stuart Cull-Candy

EVERYONE, surely, knows of the inebriating effects of alcohol, yet few would claim to understand exactly how this drug causes intoxication. One experimental approach to the problem has been to breed rats or mice selected for differences in alcohol sensitivity, with the aim of identifying the genes responsible and their products. On page 356 of this issue<sup>1</sup>, Korpi *et al.* describe a gene mutation in one such rat line. The rodent in question is the alcohol-non-tolerant (ANT) rat, a hapless animal that is highly susceptible to the impairment of postural reflexes by alcohol and benzodiazepines. The mutation is a single amino-acid change in one subunit of a receptor for the ubiquitous inhibitory neurotransmitter,  $\gamma$ -aminobutyric acid (GABA).

To appreciate the significance of this finding it is necessary to recall something of the increasingly complex world of GABA receptors. The principal inhibitory effect of GABA, an increase in chloride conductance of the neuronal membrane, is mediated by the 'GABA<sub>A</sub> receptor'. Unfortunately, this term is now hopelessly imprecise. Like other ligand-gated ion channels, GABA<sub>A</sub> receptors are thought to contain five heterologous subunits. In this case there is certainly no shortage of subunit combinations from which to choose; genes encoding more than a dozen distinct subunit proteins belonging to five classes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\rho$ ) have been identified. Functional artificially expressed GABA channels can be formed from receptor subunits of one class alone (homomeric) or by subunits of different classes in combination. Importantly, the pharmacological and biophysical properties of these receptors depend critically on their subunit composition. Whereas homomeric receptors will respond to GABA, the presence of a  $\gamma$  subunit in conjunction with an  $\alpha$  and  $\beta$  is required for the characteristic potentiation of GABA responses by benzodiazepines such as diazepam. In turn, the precise nature of the response to a range of different benzodiazepine ligands depends largely on the nature of the associated  $\alpha$  subunit.

A striking example of this dependence can be found in the case of the  $\alpha 6$  subunit, which, uniquely among GABA<sub>A</sub> receptor subunits, is restricted to a single neuron type within the central nervous system — the cerebellar granule cell<sup>2,3</sup>. Recombinant receptors containing this subunit have an extremely low

affinity for benzodiazepine agonists (including diazepam) but a high affinity for Ro15-4513 (ref. 2), one of a class of benzodiazepine-receptor ligands that weakly inhibit rather than potentiate the action of GABA. This unusual profile results from the presence of an arginine residue at position 100 in the extracellular amino-terminal domain of the  $\alpha 6$

Ro15-4513 binding comparable to that seen with cerebellar tissue from ANT rats, and give rise to GABA-induced currents that can be potentiated by diazepam.

The attractive implication is clear: in ANT rats the excessive motor-impairment caused by benzodiazepines reflects an anomalous response of their  $\alpha 6$ -containing cerebellar GABA<sub>A</sub> receptors to these drugs. This is indeed what the authors propose — the first suggestion that a specific phenotype results from a point mutation in a GABA<sub>A</sub> receptor subunit. These findings clearly reinforce the importance of the amino-

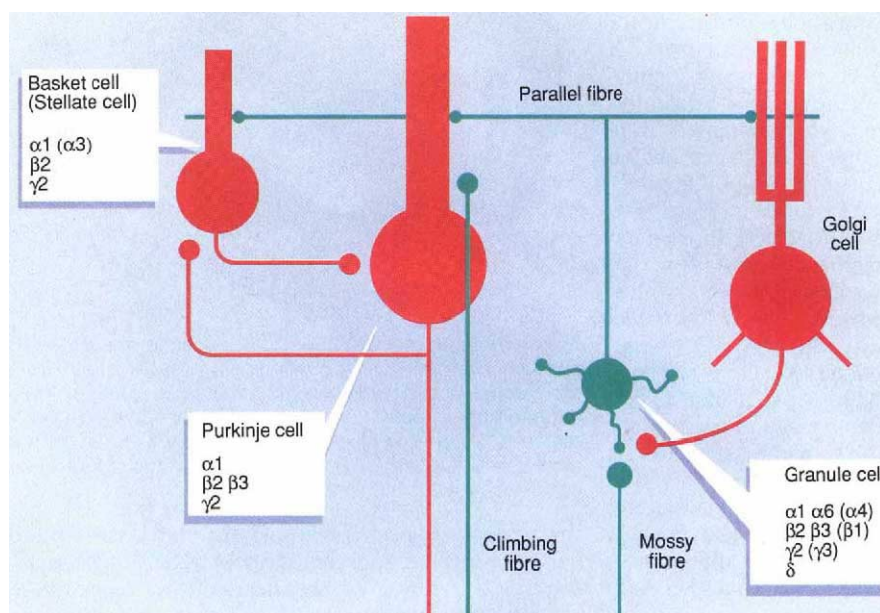


Diagram of the intrinsic neurons of the cerebellum and their expression of GABA<sub>A</sub> receptor subunit mRNAs as detected by *in situ* hybridization in the adult rat<sup>3</sup>. Inhibitory GABAergic cells are shown in red, excitatory pathways and cells in green. The granule cell receives excitatory input from mossy fibres and axo-dendritic inhibitory input from Golgi cells. The mRNA species shown in parentheses are expressed at lower levels. Only cerebellar granule cells express  $\alpha 6$ .

protein, a position occupied by a histidine residue in most other  $\alpha$  subunits<sup>4</sup>. The peculiar properties of  $\alpha 6$ -containing receptors provide a convenient hallmark for their identification — 'diazepam-insensitive Ro15-4513 binding'. Although binding of this type is found in the cerebellum of normal rats, it is absent from most ANT rats<sup>5</sup>.

Korpi *et al.*<sup>1</sup> have now identified the reason for this absence — a point mutation in the gene encoding  $\alpha 6$  which alters the very residue previously identified as governing benzodiazepine affinity. In this case, however, the switch is not to a histidine (as found in  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 5$ ) but to a glutamine. This change does not alter the expression of the receptor but it does drastically alter its functional properties. Unlike receptors containing the wild-type  $\alpha 6$ , those containing the modified  $\alpha 6$  subunit exhibit diazepam-sensitive (not insensitive)

acid residue at position 100 of  $\alpha$  subunits in controlling the binding of benzodiazepine agonists<sup>4</sup>. Consideration of the role of  $\alpha 6$ -containing GABA receptors in the cerebellar circuits that control motor reflexes requires more caution. Although the evidence is tantalizingly suggestive, there is as yet no firmly established link between mutation of the  $\alpha 6$  gene and the altered behaviour of ANT rats.

This caveat need not prevent speculation. As it seems reasonable to think that benzodiazepine-induced postural impairment results from enhanced GABAergic transmission, the work of Korpi *et al.* might be taken to mean that the  $\alpha 6$  subunit forms part of a functional synaptic receptor. This is not a trivial conclusion. At present relatively little is known about the location within neurons of GABA receptors containing specific subunits, yet the need for such informa-



tion is manifest. Various studies have revealed the presence in granule cells of a bewildering array of GABA<sub>A</sub>-subunit messenger RNAs. In cultured cells 14 mRNA species have been detected, albeit in different abundances<sup>6</sup>. *In situ* hybridization of adult rat cerebellum presents a picture that is only slightly less confusing. Here the principal granule-cell mRNAs encode  $\alpha 1$ ,  $\alpha 6$ ,  $\beta 2$ ,  $\beta 3$ ,  $\gamma 2$  and  $\delta$  subunits, with a lesser contribution from  $\alpha 4$ ,  $\beta 1$  and  $\gamma 3$  (ref. 3).

The immediate hope of determining how these subunits actually come together to form receptors rests with immunological methods. For example, from immunoprecipitation studies it seems that most but not all GABA<sub>A</sub> receptors contain only a single type of  $\alpha$  subunit, and specifically that  $\alpha 6$ , although not found in combination with other  $\alpha$  subunits, is present in two distinct populations of receptor<sup>7</sup>. Because both  $\alpha 1$  and  $\alpha 6$  immunoreactivity is present at synapses between Golgi cell terminals and granule dendrites<sup>8</sup>, these data collectively imply that GABA released from Golgi cells (the primary GABAergic input to granule cells; see figure) may act on several GABA<sub>A</sub> receptor types. The reasons for such heterogeneity and its normal functional consequences remain to be established — this is a challenging task for electrophysiologists.

What of the relationship between  $\alpha 6$  and alcohol? Despite claims arising at the time of the cloning of this subunit, it seems unlikely that  $\alpha 6$  has a unique role in the action of alcohol or the antagonism of its behavioural effects by Ro15-4513. In the case of ANT rats, the mutation in  $\alpha 6$ , which may underlie their susceptibility to motor impairment by benzodiazepines, could reflect gene fixation and need not be related to the alcohol sensitivity for which these animals were selected. Recently, attention has been directed to the  $\gamma 2$  GABA<sub>A</sub> subunit and in particular the alternative-

ly spliced form,  $\gamma 2L$ , which bears a site for phosphorylation by protein kinase C on an 8-amino-acid insert<sup>9</sup>. The presence of  $\gamma 2L$  (ref. 10) and the integrity of its phosphorylation site<sup>11</sup> appear to be essential for modulation of expressed receptors by ethanol. It now seems that, rather than GABA<sub>A</sub> receptors themselves, one determinant of alcohol

sensitivity may lie in the enzymatic machinery of phosphorylation processes which act upon them. A sobering thought indeed. □

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## MANTLE CONVECTION

# A natural mineral filter

Ulrich Christensen

WHETHER the Earth's mantle is a homogeneous chemical mix or is divided into compositional layers has exercised the talents of geochemists for many years; a strong case can be made either way. For geodynamicists, the related (but not completely equivalent) question is whether convection takes in the mantle as a single layer or occurs in several layers. It has usually been assumed that compositional layering, if it exists, is a relic of differentiation in the very early days of the Earth, but S. Weinstein now suggests<sup>1</sup> that layering may have developed through convection over geological times. With numerical modelling, he shows that the physical phase change associated with the known seismic discontinuity at 660 km depth could act as a chemical filter on whole-mantle convection: chemically dense rock could pass down into the lower mantle, and buoyant rock up into the upper mantle. The phase change would inevitably lead to compositional layering.

Most of the discussion on layering of the mantle has centred around the 660-km discontinuity, where the seismic velocities and the density increase by about 10 per cent over an interval of a few kilometres. Either a change in composition or a structural phase transition could be the cause of the discontinuity. It might be a combination of both, but previously no convincing explanation has been provided for why, in this case, phase and material boundaries should coincide.

## Transformation

High-pressure experiments performed over the past decade have shown that all known major features of the 660-km discontinuity are explained by the isochemical transformation from the  $\gamma$ -spinel phase of olivine (Mg,Fe)<sub>2</sub>SiO<sub>4</sub> into a mixture of perovskite (Mg,Fe)SiO<sub>3</sub> and magnesiowüstite (Mg,Fe)O. The experiments also indicate that the transition to the high-pressure phase is endothermic. Still, a slight change in composition equivalent to a density difference of a few per cent

juxtaposed on the phase change cannot be excluded. The uncertainties in the thermodynamic properties for lower-mantle minerals are sufficiently large that a range of compositions seems compatible with the seismically determined properties of the lower mantle<sup>2</sup>.

An endothermic phase transition would affect convection, because the transition would occur at greater depths in cold subducting lithospheric slabs, and higher in rising warm plumes. Such deflections would create gravitational forces that counteract the buoyancy forces driving convection. An improbably strong degree of endothermicity was initially thought necessary for generating layered convection<sup>3</sup>, whereas models that are more realistic, with a revised estimate for thermal expansion, conclude that convection may be at least episodically layered, with occasional excursions across the boundary possible<sup>4,5</sup>.

In his new numerical experiments, Weinstein demonstrates that the additional (negative) buoyancy of chemically dense rock embedded in a descending convection current in the upper mantle can give the extra kick necessary to get the material into the lower mantle, as if through a trap door that stops ordinary rock. And dense rock in an upwelling plume in the lower mantle is filtered out at the phase transition, while material of background composition is just able to pass into the upper mantle. Any variations in a mantle that is on average the same above and below the phase boundary will lead, over a few overturn cycles, into a chemically layered system with a sharp compositional gradient at the phase boundary.

Models of a chemically layered mantle have been most popular with geochemists, who wanted separate reservoirs of material to explain the diversity of isotope ratios found in mantle-derived basalts. Most geophysicists preferred a homogeneous mantle and unlayered circulation, mainly for reasons of simplicity. Between these extremes, several semi-layered, compromise models have been proposed<sup>6,7</sup>, but none has yet been

1. Korpi, E. R., Kleingoor, C., Kettenmann, H. & Seeburg, P. H. *Nature* **361**, 356–359 (1993).

2. Lüddens, H. et al. *Nature* **346**, 648–651 (1990).

3. Laurie, D. J., Seeburg, P. H. & Wisden, W. *J. Neurosci.* **12**, 1063–1076 (1992).

4. Wieland, H. A., Lüddens, H. & Seeburg, P. H. *J. Biol. Chem.* **267**, 1426–1429 (1992).

5. Uusi-Oukari, M. & Korpi, E. R. *J. Neurochem.* **54**, 1980–1987 (1990).

6. Bovolin, P., Santi, M. R., Puia, G., Costa, E. & Grayson, D. *Proc. natn. Acad. Sci. U.S.A.* **89**, 9344–9348 (1992).

7. Gillard, N. P., Quirk, K., Ragan, C. I., Whiting, P. & McKernan, R. M. *Fidia Res. Fdn Symp.*, Cambridge, 8–10 September 1992.

8. Baude, A., Sequier, J.-M., McKernan, R. M., Olivier, K. R. & Somogyi, P. *J. Neurosci.* **51**, 739–748 (1992).

9. Whiting, P., McKernan, R. M. & Iversen, L. L. *Proc. natn. Acad. Sci. U.S.A.* **87**, 9966–9970 (1990).

10. Wafford, K. A. et al. *Neuron* **7**, 27–33 (1991).

11. Wafford, K. A. & Whiting, P. J. *FEBS Lett.* **313**, 113–117 (1992).

shown to be dynamically feasible. Weinstein's work is the first demonstration that chemical layering can develop in a mantle without strict convective layering.

Is it relevant to the Earth? Weinstein shows that only a narrow range of properties of the phase transition gives the required imperfect layering. Whether the Earth's mantle falls in this range is uncertain, but it is not unlikely. The most important source of mantle heterogeneity that we know is the subduction of basaltic oceanic crust. Turned into eclogite it becomes denser than mantle peridotite and so may be expected to sink more readily into the lower mantle than the rest of the lithosphere.

### Buoyancy

But the story is more complex, because the difference in composition between basaltic and peridotitic rock affects the phase equilibria, which may render basaltic material buoyant near the 660-km discontinuity. This has led Ringwood and Irifune to speculate<sup>7</sup> that it may be trapped in a transitional layer between the upper and lower mantle. On the other hand, the subducted crust forms a thin sheet which should stick tightly to the rest of the lithosphere owing to its high viscosity, so that it may not separate readily from the rest of the subducted slab<sup>8</sup>.

Weinstein's work is one of several studies suggesting that the 660-km phase boundary leads to exciting dynamical behaviour, giving neither layered nor whole-mantle convection. Seismic pictures of subducting slabs that penetrate into the lower mantle in some places but stagnate in a transition zone in others<sup>9</sup> accord with these calculations. With increasing sophistication allowing a closer representation of the real conditions of the Earth's mantle, the hitherto speculative, complex concepts of semi-layered convection<sup>6,7,10</sup> will soon be put to the test. □

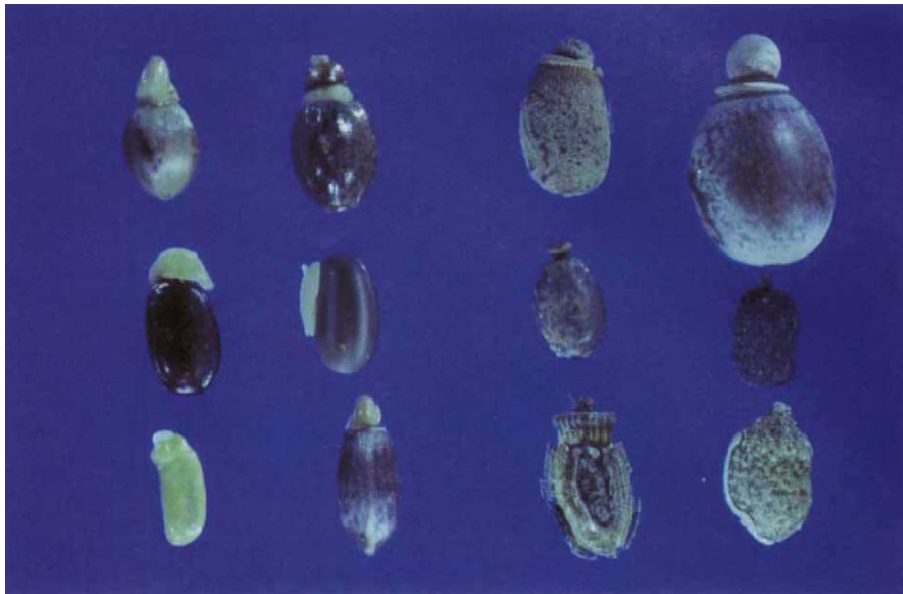
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## How to get carried away

Peter D. Moore

DARWIN<sup>1</sup> considered that in nature there is virtually "no limit to the amount of profitable diversification of structure and therefore no limit to the number of species which might be produced". Despite the apparently infinite number of possibilities, however, there are occasions when nature comes up with the same answer to a problem that is faced by very different species. The morpho-

Many plant seeds have eliasomes that attract ants and lead to their being carried off to nests for consumption or storage<sup>3</sup>, and Willson<sup>4</sup> has pointed out that most of the plants that show this feature are short and therefore benefit from the advantages of wider dispersal that the energy investment in the eliasome secures. Once the oily eliasome has been eaten, the seeds are discarded



Seeds of ant-dispersed plants (six on left) and stick-insect eggs (six on right); from ref. 2.

logical resemblance between certain plant seeds and the eggs of stick-insects, now pointed out by L. Hughes and M. Westoby in *Functional Ecology*<sup>2</sup>, must be one of the most remarkable convergences on record, and one in which the precise nature of the selective forces remains obscure.

According to Hughes and Westoby only about 5% of the eggs of the 2,500 species of stick insect (order Phasmatoidea) have been described, but about half of those that have been documented have a swollen 'capitulum' attached to the operculum, or lid, through which the nymph emerges. The precise function of the capitulum has never been determined, but when it was first described in 1898 it was pointed out that it resembles the appendage carried by castor oil seeds, now known to provide an oily reward to ants who disperse the seeds while exploiting the food reserves in the so-called eliasome. Could the capitulum of stick-insect eggs serve a similar function to the eliasome of some plant seeds? That begs the question of whether the enhanced dispersal of eggs is really such an advantage to a mobile stick insect as it is to a sessile plant.

and may then germinate in their new locations. It is also possible that being cast upon an ant midden brings nutritional advantages to the established seedling, but this is disputed<sup>5</sup>. Even if this were the case for seedlings, it is unlikely to be of great benefit to stick-insect nymphs.

Hughes and Westoby show that ants do select both eggs and seeds in which the capitulum, or the eliasome, are present in preference to those from which these appendages have been removed. Stick-insect eggs taken to nests are subsequently buried, raising the question of whether the nymphs can get back to the surface after emergence. Experimental burial of eggs confirmed that 98% of nymphs emerging at 6 cm depth and even 11% of those emerging at 12 cm depth arrived safely back at the surface. One wonders, however, if the upwardly mobile nymphs would have been so successful when ascending through an active ants' nest.

A further possible advantage associated with ant burial examined by Hughes and Westoby is the protection it provides the eggs from parasitism. The proportion of non-parasitized eggs in a sampled profile rose from about 20% in the

- Weinstein, S. *Earth planet. Sci. Lett.* **113**, 23–40 (1992).
- Bukowski, M. S. T. & Wolf, G. H. *J. geophys. Res.* **95**, 12583–12593 (1990).
- Christensen, U. & Yuen, D. A. *J. geophys. Res.* **90**, 10291–10300 (1985).
- Machetel, P. & Weber, P. *Nature* **350**, 55–57 (1991).
- Honda, S., Yuen, D. A., Balachandrar, S. & Reuteler, D. *Science* (in the press).
- Silver, P. G., Carlson, R. W. & Olson, P. A. *Rev. Earth planet. Sci.* **16**, 477–541 (1988).
- Ringwood, A. E. & Irifune, T. *Nature* **331**, 131–136 (1988).
- Richards, M. A. & Davies, G. F. *Geophys. Res. Lett.* **16**, 831–834 (1989).
- van der Hilst, R., Engdahl, R., Spakman, W. & Nolet, G. *Nature* **353**, 37–43 (1991).
- Anderson, D. L. *Theory of the Earth* (Blackwell Scientific, Boston, 1989).



surface litter to over 40% at a depth of 5–10 cm, so this could confer the required survival advantage.

There is one further aspect of burial, familiar to students of ant-dispersed seeds<sup>6</sup> but not considered by Hughes and Westoby, that could also provide a strong selective pressure favouring burial of insect eggs, namely escape from fire. Temperature profiles in grassland fires in the tropics and sub-tropics usually display peak values in the surface litter of the soil, which is precisely where the eggs of the stick insects would lie if they were not buried by ants. The temperature a few centimetres down the soil profile, however, remains relatively low and would permit the survival of the eggs as well as of the ant-dispersed seeds. Fire avoidance by burial could well prove to be a survival mechanism worth the investment of energy in a capitulum and the inherent risks to a nymph in an active ants' nest.

Whatever the selective pressures that have led to this remarkable morphological convergence between plant seeds and insect eggs, nature has not needed to resort to its infinite potential for diversification. The common evolutionary answer to the problem has proved to be the development of a small, oily knob. □

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1. Darwin, C. *The Origin of Species* 6th edn (John Murray, London, 1872).
2. Hughes, L. & Westoby, M. *Functional Ecol.* **6**, 642–648 (1992).
3. Beattie, A. J. *The Evolutionary Ecology of Ant-Plant Mutualisms* (Cambridge University Press, 1985).
4. Willson, M. F. in *Seeds: The Ecology of Regeneration in Plant Communities* (ed. Fenner, M.) 61–85 (CAB Int., Wallingford, 1992).
5. Bond, W. J. & Stock, W. D. *Oecologia* **81**, 412–417 (1989).
6. Bennett, A. & Krebs, J. *Trends Ecol. Evol.* **2**, 291–292 (1987).

## GEOMAGNETISM

# About turn for reversals

Catherine Constable

THE idea that the Earth's magnetic field during geomagnetic reversals tends to sweep down a line through the Americas<sup>1–3</sup>, or else along the antipodal line through East Asia and Australasia, has stirred up a lot of debate. If true, it suggests that the Earth's magnetic field, generated in the Earth's core, is somehow controlled by the overlying mantle, presumably by regional variations of temperature, composition or topography at the core–mantle boundary. On page 342 of this issue<sup>4</sup>, McFadden *et al.* apply a specially devised statistical test to the data that support this notion of preferred paths, and argue that they could be a product of the restricted distribution of sampling sites available to geomagnetists.

Identifying the transitional field over the 10,000 years it takes for a reversal to occur involves finding sediments or volcanic rocks laid down over those years and measuring the declination and inclination of the frozen-in (remanent) magnetization. From these data at a single site, one can determine the path of the geomagnetic pole. The fact that data from several sites indicated the same path has led to the suggestion that the field remains largely dipolar during a transition (although it is difficult to reconcile this with the observation that both antipodal paths can occur during a single reversal); the fact that the same paths were repeated time and again suggests that some form of mantle control is exercised — without it, the fluid

core seems unlikely to have a preferential non-axisymmetric orientation. Exciting support for this idea came in the form of correlations of the paths to models of fluid flow at the core surface and to variations in seismic velocity in the lower mantle.

But the interpretation of the reversal paths is controversial on a number of counts. First, it is debated whether the observations genuinely show a preference for particular longitudinal regions or simply reflect the statistics of the inevitably small sample<sup>5–7</sup>. Second, should one expect to see a uniform distribution over the globe, given the uneven distribution of sites at which

reversal records are available<sup>5,6</sup> (Fig. 1)? Third, there is the issue of whether the records have sufficient resolution to recover the field structure during reversals<sup>8,9</sup>; the magnetic remanence in many sedimentary records reflects an average geomagnetic field direction over several thousands of years and may be dominated by the relatively high intensity of the field after it returns to its full polarity state.

McFadden *et al.*<sup>4</sup> have devised a more specific, and so more powerful, statistical test than any other so far invoked. Starting from scratch, they come to two conclusions. First, that the data, sparse though they are, genuinely show clumping — the measured pole longitudes are preferentially grouped within two antipodal bands. But second, they show that, as others have hinted before<sup>5,6</sup>, there is a tendency for the sites at which reversals are sampled to lie 90° either side from their associated virtual geomagnetic pole paths.

A possible origin of this tendency is illuminated in a new paper by Egbert<sup>10</sup>, who has investigated the effect of the transformation in spherical coordinates, used to find the virtual geomagnetic poles, on simple, statistically homogeneous palaeomagnetic directions. Except in the special case where the field distribution is entirely dipolar, the distribution of longitudes is always peaked  $\pm 90^\circ$  from the sampling longitude. Unless the distribution of sampling sites is uniform, one should not expect the distribution of pole longitudes to be uniform.

So is there any significant observation left to explain? Of the two distributions examined by McFadden *et al.* — the longitudes of the measured poles and their longitudes relative to the measuring site — the violation of the null hypothesis is stronger for the former than for the latter (the probabilities differ by almost two orders of magnitude): the

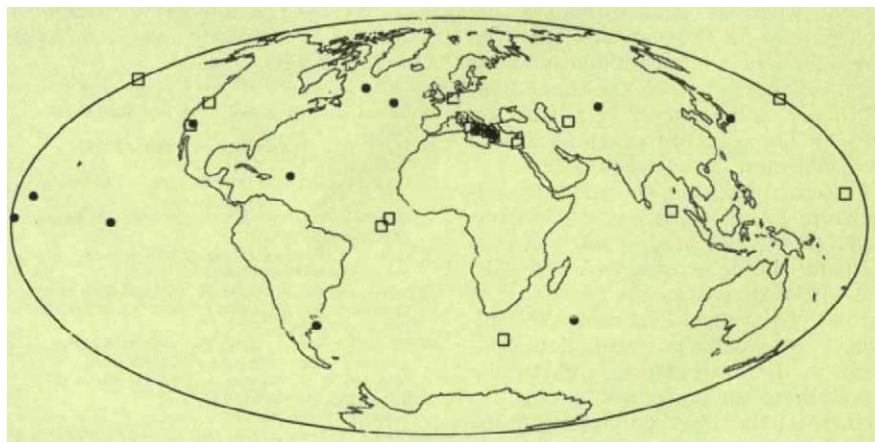


FIG. 1 Sites of geomagnetic reversal records for the past 12 million years<sup>6</sup>. Open squares represent reversal records whose equatorial crossings for the transition path lie  $90^\circ \pm 30^\circ$  from the site longitude. Solid symbols correspond to records without this bias.

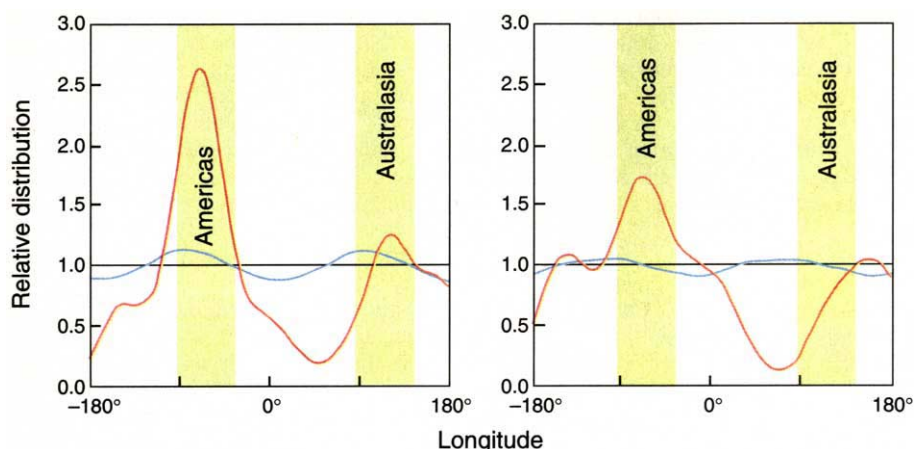


FIG. 2 *a*, Observed distribution of virtual geomagnetic poles (at equatorial crossings) during reversals (red line), normalized to that expected for a uniform distribution (solid line) and compared with the distribution predicted by Egbert's isotropic statistical model<sup>10</sup> for local field variability (blue line). *b*, Same as *a* for those sites that show no tendency to give virtual poles 90° away.

site locations may not be the only factor influencing the result. Egbert's calculations allow us to compute the expected distribution of pole longitudes for any given site distribution, supposing that a reasonable assumption can be made regarding the statistics of the variability in the magnetic field with time. Unfortunately, the correct statistics are part of what we would like to observe by studying reversal paths; however, a statistical model corresponding to isotropic directional variability in local field coordinates is probably reasonable for reversals, during which the dipole (the most strongly anisotropic part of the field) is much diminished. As can be seen in Fig. 2*a*, the bias expected as a result of site distributions seems small in comparison with the overall signal, but the distribution does peak in approximately the right places. Egbert also predicts that the bias to site longitude  $\pm 90^\circ$  should be more prevalent at low latitudes, a feature not observed (Fig. 1). The apparent bias to American longitudes is still present when all sites exhibiting any tendency to  $\pm 90^\circ$  bias are removed (Fig. 2*b*), although the antipodal band is effectively squashed by this operation, and there is no longer a correlation between the observed peaks and those predicted from the site distribution.

These two latest papers and the trends shown in Fig. 2 effectively address two of the problems regarding the preferential paths during geomagnetic reversals. The observations are unlikely to arise as a result of statistical fluctuations in sampling from a uniform distribution; and, although there are effects due to site distribution, they are not enough to account for the observed biases. On the third issue, namely whether sediments accurately record the magnetic paths during reversals, it has been shown that for several examples, the apparent paths

can be obtained simply by averaging the direction of the stable field before and after the reversal<sup>8,11</sup>. Furthermore, from lava flows I have found<sup>12</sup> a bias in the preferred longitudes of the virtual geomagnetic poles of the stable field. Indeed, the preferred longitudes are the same as those seen in the reversal records.

Although this may point to some kind of mantle control over the geodynamo, it leaves one doubting the sedimentary records of reversals. But lava flows are not subject to the same kind of smoothing as are sediments, and where these record reversals, they too sometimes show a longitudinal bias<sup>13,14</sup>. The intermittency of volcanic records means that these do not give a continuous and well dated record, but future correlation between nearby sedimentary and lava palaeomagnetic data for the same reversal may clarify this otherwise confusing puzzle. □

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1. Clement, B. M. *Earth planet. Sci. Lett.* **104**, 48–58 (1991).
2. Tric, E. *et al. Phys. Earth planet. Inter.* **65**, 315–336 (1991).
3. Laj, C., Mazaud, A., Weeks, R., Fuller, M. & Herrero-Bervera, E. *Nature* **351**, 447 (1991).
4. McFadden, P. L., Barton, C. E. & Merrill, R. T. *Nature* **361**, 342–344 (1993).
5. Valet, J. P., Tueloika, P., Courtillot, V. & Meynadier, L. *Nature* **356**, 400–407 (1992).
6. Laj, C., Mazaud, A., Weeks, R., Fuller, M. & Herrero-Bervera, E. *Geophys. Res. Lett.* **19**, 2003–2006 (1992).
7. Laj, C., Mazaud, A., Weeks, R., Fuller, M. & Herrero-Bervera, E. *Nature* **359**, 111–112 (1992).
8. Langereis, C. G., van Hoof, A. A. M. & Rochette, P. *Nature* **358**, 226–229 (1992).
9. Weeks, R., Fuller, M., Laj, C., Mazaud, A. & Herrero-Bervera, E. *Geophys. Res. Lett.* **19**, 2007 (1992).
10. Egbert, G. *Geophys. Res. Lett.* **19**, 2353–2356 (1992).
11. Jackson, A. *Nature* **358**, 194–195 (1992).
12. Constable, C. *Nature* **358**, 230–233 (1992).
13. Hoffman, K. A. *Nature* **354**, 273–277 (1991).
14. Hoffman, K. A. *Nature* **359**, 789–794 (1992).

## Blood and tears

HAPPY and hopeful people heal faster than miserable ones. They may even resist infection in the first place. The mechanism, says Daedalus, must be hormonal. Thus in rats, the immune system is strongly sabotaged by removing the pituitary gland, but can be restored by injections of the hormone prolactin. So when we are happy our brain or pituitary must release some endorphin of euphoria into the blood, where it stimulates the circulating lymphocytes and phagocytes. Conversely, in our blacker moods the brain must saturate the blood with a negative endorphin. It inhibits the immune system and invites wandering infections to put us out of our misery.

This has implications for blood transfusion. A litre of blood from some depressed or suicidal personality should make the luckless recipient feel even worse than before. An equivalent litre from an irrepressible optimist should infuse him with hope and encouragement. Some transfusion services buy their blood. They tend to attract down-and-outs and alcoholics: no-hopers with nothing else to sell. Their abject mental state must be bad for the patients. The British service uses unpaid volunteers, high-minded citizens whose mood should be much more therapeutically bracing. DREADCO psychiatrists are now conducting personality tests on prospective blood donors, identifying beforehand the stressed businessmen and tortured intellectuals, the cheerful extraverts and contented couples. They are even throwing wild parties and uninhibited orgies for selected groups of donors, so as to extract their blood during the period of maximum euphoric abandon.

The resulting blood samples will be medically tested in a standard double-blind trial. If happy blood is indeed better for patients than miserable blood, transfusion services everywhere will adopt Daedalus's methods. Blood donation may be transformed from a civic duty to an orgiastic delight. Meanwhile, DREADCO pharmacologists are analysing the samples for the hormones responsible. Like the blood itself, they may come in many different varieties, each best suited to its original donor. If so, after any great triumph or surge of happiness, it would be sensible to give a generous volume of blood, and store it for yourself. In a subsequent illness, disaster or fit of depression, you could have it transfused back again. What better and safer tranquillizer or mood-lifter than the one used by your own body? Even an extreme manic-depressive might be neatly stabilized by extracting blood during his manic phase and re-injecting it during his depressive one.

David Jones



## Earliest *Homo* not proven

SIR — A closer look at the morphological evidence on which Hill *et al.*<sup>1</sup> assert that the temporal bone KNM-BC 1 from the Chemeron formation near Lake Baringo in Kenya represents the earliest member of the genus *Homo* casts serious doubt on their claim. The specimen was originally identified by Martyn and me as that of a hominid of indeterminate genus and species<sup>2</sup>, because, on the evidence of this temporal bone alone, it was impossible to assign the specimen firmly to either of the most widely recognized genera of the Hominidae, *Australopithecus* or *Homo*. Although most features of the Chemeron temporal suggested australopithecine affinities, a good proportion suggested an affinity with *H. habilis*<sup>2</sup>.

Hill *et al.*<sup>1</sup> conclude that the Chemeron temporal shares two features which "appear to be unique to members of the *Homo* clade". For the first feature, "medially situated temporomandibular joint fossa", they offered no metrical or topographic statement as to how far medial the fossa lies. The marked variability in medial or lateral positioning of the mandibular fossa was noted by Weidenreich<sup>3</sup> and me<sup>4,5</sup>. The trait appears to reflect both the degree of expansion of the brain (ref. 3, pp 50–51) and the size of the mandibular condyle (ref. 5, pp 110–111). In great apes and robust australopithecines, the medial half or less of the articular tubercle is fronted by infratemporal surface; the lateral half or more of the fossa lies beyond the plane of the side wall of the calvaria. In these forms the frontal part of the brain is little expanded, but the mandibular condyle is large. In *A. africanus*, which has a relatively slightly expanded frontal part of the brain, but a smaller condyle, a greater proportion of the fossa is fronted by brain-case. This proportion is two-thirds to three-quarters in Transvaal *A. africanus* crania such as Sts 5, Sts 19 and MLD 37/38, and the same proportions apply in the Olduvai *H. habilis* crania OH 24 and OH 13. In other words, the fossa is just as far medially situated in *A. africanus* as in *H. habilis*<sup>5</sup>. A still greater proportion of the fossa is medial in *H. erectus* and *H. sapiens* (80–100%)<sup>5</sup>. Thus, the medial position of the mandibular fossa must be removed from the short list of temporal features which have been said to distinguish between *Homo* and *Australopithecus*.

This leaves only what Hill *et al.* call (erroneously) the sharp petrous crest. The petrous or petrosal crest is Weidenreich's name for a feature of the tympanic plate on the basis cranii externa<sup>3</sup>; what Hill *et al.* mean is the superior

margin of the petrous pyramid<sup>4,6</sup>. Originally it was pointed out that this feature in the Chemeron temporal resembles those of Olduvai hominid 5 (*A. boisei*), Sts 5 (*A. africanus*) and modern man, and contrasts with that of *H. erectus*, including OH 9 (ref. 2). Falk and Baker in their Scientific Correspondence<sup>7</sup> have correctly reminded us of the resemblance of KNM-BC 1 to OH 5, and at the same time have ingeniously devised a method for the metrical expression of the form of the petrous pyramid as a whole; and Rightmire has confirmed the "blunt and flattened superior margin of the petrous pyramid" in OH 9 (ref. 6, p. 70). The form of the superior margin in some hominid taxa can be summarized as follows (see ref. 5, p. 767): *A. africanus* acuminate, *A. robustus*?, *A. boisei* acuminate, *H. habilis* acuminate, *H. erectus* blunt, *H. sapiens* acuminate. This feature is thus variable within the genera *Australopithecus* and *Homo* and, despite the claim of Hill *et al.* that a sharp margin appears to be unique to the *Homo* clade, the trait in fact fails to distinguish between the two genera.

In sum, neither of the two anatomical features distinguishes between the *Homo*

clade and some other hominids of the genus *Australopithecus*. Therefore the claim of Hill *et al.* that the Chemeron temporal represents the earliest *Homo* is disconfirmed and the bone must remain, as we originally concluded<sup>2</sup>, a hominid specimen of indeterminate genus and species. Although other evidence<sup>8,9</sup> points to the probable existence in East Africa of the genus *Homo* by 2.4–2.2 Myr before present, it is certainly invalid to claim that the Chemeron temporal extends the origin of the *Homo* clade "more surely by about half a million years".

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- Hill, A., Ward, S., Delno, A., Curtis, G. & Drake, P. *Nature* **355**, 719–722 (1992).
- Martyn, J. E. & Tobias, P. V. *Nature* **215**, 476–480 (1967).
- Weidenreich, F. *Pal. Sinica New Ser.* **D10**, 1–484 (1943).
- Tobias, P. V. *Olduvai Gorge* **2** (Cambridge University Press, 1967).
- Tobias, P. V. *Olduvai Gorge* **4** (Cambridge University Press, 1991).
- Rightmire, G. P. *The Evolution of Homo erectus* (Cambridge University Press, 1990).
- Falk, D. & Baker, E. *Nature* **358**, 289–290 (1992).
- Howell, F. C. *et al. J. hum. Evol.* **16**, 665–700 (1987).
- Boaz, N. T. A. *Rev. Anthropol.* **8**, 71–85 (1979).

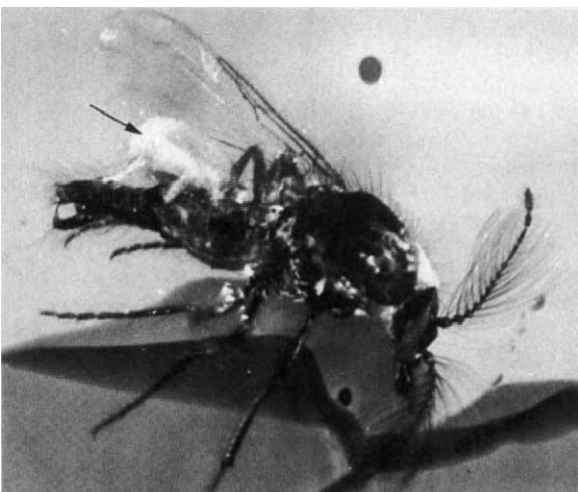
## Animal–animal parasitism

SIR — Parasitic mites found on the dorsa of their biting midge hosts in Cretaceous Canadian amber (70–80 million years old) constitute the earliest fossil evidence of terrestrial animal–animal parasitism. The mouthparts of the parasites are still attached to the integument of their hosts, leaving little doubt as to their parasitic nature.

Examples of actual parasitism in the fossil record are rare. We have ob-

served four examples (of a total of 81 specimens) of mite parasitism of biting midges (Ceratopogonidae: Diptera) in Canadian amber collected from the Foremost formation (Judith River group) near Grassy lake, Alberta. Radiometric dating indicated an age of 70–80 million years for these deposits<sup>2</sup>.

The four biting midges all belong to extinct genera in the subfamily Ceratopogoninae. They are all quite small, ranging from 896 to 1,540  $\mu\text{m}$  in length. The mites are all larval stages and ranged from 158 to 391  $\mu\text{m}$  in length. Three of them are still attached to the backs of their biting midge hosts. The mite in the fourth specimen is detached from its host and probably had only recently become associated with the biting midge. The other three mites are clearly in the act of feeding on the midges, since not only are their mouthparts still in contact with the hosts, but their bodies are partly engorged (see figure). Three of the mites belong to the family Erythraeidae, while



Partially engorged mite of the Phalanx Trombidia (arrow) in feeding position on the back of a biting midge (Ceratopogonidae) in 70–80 million-year-old Canadian amber.

the fourth is in the Phalanx Trombidia and possibly a member of the family Microthrombidiidae. Representatives of both of these mite groups have been noted on extant adult Ceratopogonidae (R. V. Southcott, personal communication).

These mites are typically parasitic only in the larval stage. Later, in the nymphal and adult stages, they live as predators on a range of invertebrates. These examples represent the earliest known fossil records of mite parasitism of invertebrates, as well as animal-animal parasitism in general. They show that biting midges were themselves the victims of parasitism as early as the late Mesozoic.

The specimens (RTMP 92.8.4, 92.8.5, 92.8.6 and 92.8.7) are deposited in the Royal Tyrrell Museum of Paleontology at Drumheller, Alberta.

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1. Boucot, A. J. *Evolutionary Paleobiology of Behavior and Coevolution* (Elsevier, Amsterdam, 1990).

2. Folinsbee, R., Baadsgaard, E. H., Cumming, G. L. & Nasrinbene, J. *Bull. Am. Ass. Petrol. Geol.* **48**, 525 (1964).

## Size segregation in powders

SIR — Jullien *et al.*<sup>1</sup> have described a computer simulation method for reorganizing stable close packings of hard spherical particles (see ref. 2). Repeated applications of this technique to a mixture of grains with different sizes cause size segregation. An important consequence of the method<sup>1</sup> is the prediction of a critical size ratio for segregation to occur — in other words, a single large impurity sphere will rise through a bed of smaller spheres only if the impurity is larger than a critical size. This threshold has not been observed in size-segregation experiments (see, for example, ref. 3), and is otherwise unexpected — given the importance of shaking-induced size segregation in industry and technology, it is therefore very important to know whether or not it really exists. To do this, we need to know to what extent the method used by Jullien *et al.*<sup>1</sup> models a real material and a real

shaking process.

Undoubtedly, real granular materials are not made from ideal, hard spherical particles but, generally, this model of granular solids has proved to be a qualitatively reliable one and has been used by many others<sup>4</sup>. It is, however, more difficult to make a reliable representation of a shaking process. Generally, 'shaking', in the context of granular materials processing, corresponds to any externally applied, cyclic, incoherent driving force that leads to fluctuating particle configurations. Crucially, during shaking, the particles in a powder experience several different dynamical regimes, for instance the 'grain inertial' and the 'quasi-static' regimes. Although it is impossible to identify all the important features of shaking, a list of essential features would certainly include both the collective nature of the process and its complexity. In other words, not only do many particles respond to the driving force simultaneously but the forces experienced by interior particles are complex functions of both the granular structure and the coupling with the applied force. Both of these features are absent from the model studied by Jullien *et al.* which, therefore, is not a realistic representation of a shaking process.

The relatively simple, sequential method of ref. 1 can certainly produce size segregation, but this does not correspond to size segregation induced by shaking. For this reason the size threshold for segregation reported by Jullien *et al.* is not relevant to real shaking experiments (for example, ref. 3) and may be of no practical importance. In fact many counterexamples, where particles of similar sizes segregate during shaking experiments, have been observed (for example, ref. 3) and simulations which include elements that attempt to model the complexity of shaking (for example, the Monte Carlo method proposed by Rosato *et al.*<sup>5</sup>) do not show any size threshold.

The computer modelling of real granular materials is a burgeoning area of research, which is of increasing scientific and technological importance. In particular, the response of powders to vibration is an area of considerable interest, so it is crucial to incorporate the essential physics in any realistic models of this process. The absence, therefore, of non-sequential (cooperative) particle motions and complexity (which are essential

features<sup>6,7</sup> of shaking processes in powders) in the work of Jullien *et al.* renders several of their conclusions inappropriate in the context of real shaken granular materials.

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## More bubbles in volcanic systems

SIR — After my recent Scientific Correspondence about bubbles in volcanic systems<sup>1</sup>, it was brought to my attention that similar arguments had been independently made by some Russian geologists, A. Steinberg, G. Steinberg and A. Merzhanov, some years ago. After some searching, I found their excellent but unfortunately poorly circulated articles<sup>2-5</sup>. They had indeed done a very similar conceptual and experimental analysis, unbeknown to myself, my colleagues, the reviewers of my manuscript and the editors of *Nature*.

Although the discovery of their prior work causes me some consternation, our independent reproduction of the effect further demonstrates the validity of the bubble overpressure effect, as well as its applicability to volcanic systems. Neither they nor we have solved all of the problems related to bubble overpressure in volcanic systems, but since the effect is clearly relevant, the application to volcanic systems can be further quantified on the basis of magma compressibility, gas solubility<sup>5</sup>, edifice inflation and leakage of volatiles by pure gas release.

I would like to (belatedly) bring this fine work to the attention of the 'western' volcanological community, and I hope that further duplication of scientific effort can be reduced in the future by closer communication and collaboration between Russian and 'western' scientists.

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- Jullien, R., Meakin, P. & Pavlovitch, A. *Phys. Rev. Lett.* **69**, 640 (1992).
- Maddox, J. *Nature* **356**, 535 (1992).
- Foo, W. S. & Bridgwater, J. *Powder Tech.* **36**, 271 (1983).
- Walton, O. T. & Braun, R. L. *J. Rheol.* **30**, 949 (1986).
- Rosato, A., Strandburg, K. J., Prinz, F. & Swendsen, R. H. *Phys. Rev. Lett.* **58**, 1038 (1987).
- Mehta, A. & Barker, G. C. *Phys. Rev. Lett.* **67**, 394 (1991).
- Barker, G. C. & Mehta, A. *Phys. Rev.* **A45**, 3435 (1992).

- Sahagian, D. & Proussevitch, A. *Nature* **359**, 485 (1992).
- Shteynberg, G., Shteynberg, A. & Merzhanov, A. *Dokl. Akad. Nauk SSSR*, **279**, 1081-1086 (1984).
- Steinberg, G., Steinberg, A. & Merzhanov, A. *Mod. Geol.* **13**, 257-265 (1989).
- Steinberg, G., Steinberg, A. & Merzhanov, A. *Mod. Geol.* **13**, 267-274 (1989).
- Steinberg, G., Steinberg, A. & Merzhanov, A. *Mod. Geol.* **13**, 274-285 (1989).

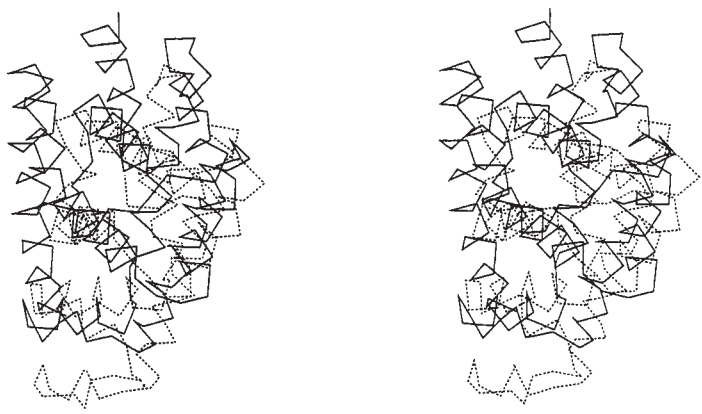


## Globin fold in a bacterial toxin

**SIR** — We have developed an algorithm for protein-structure comparison by alignment of distance matrices<sup>1</sup> and have performed an all-against-all comparison of more than 150 different protein tertiary structures in the Protein Data Bank. As a result, we have made the startling discovery that the membrane-insertion domain of the bacterial toxin colicin A (ref. 2) closely resembles the globin fold: six  $\alpha$ -helices (and one turn of  $3_{10}$ -helix) are arranged similarly in space, the packing between the helices is similar as well as the sequential order in which the chain threads through the helices (Fig. 1). The domain of colicin A thus joins the structural class that so far has consisted of the globin and phycocyanin families.

Despite similar folds, the three protein families (globins, phycocyanins and colicin A) lack significant sequence similarity, bind different cofactors (or none), and are involved in very different biological functions (oxygen transport,

**FIG. 2** Stereo view of superimposed  $\alpha$  traces of colicin A and myoglobin (shown dashed). The amino terminus of myoglobin is at the upper right, that of colicin A at the lower left.



photosynthesis, ionophoric activity). The fact that globins and phycocyanins are no longer structurally unique clearly calls for a re-evaluation of their proposed evolutionary kinship<sup>3</sup>. A structural analysis shows that colicin A is as similar to either globins or phycocyanins as they are to each other, both in terms of overall topography of the fold and in terms of side-chain packing (Fig. 2).

Colicin A kills sensitive cells by forming pores in the cytoplasmic membrane. The part of colicin A that has been solved crystallographically<sup>2</sup> is a proteolytically cleaved carboxy-terminal fragment (residues 389–592), which is water soluble and has ionophoric properties similar to those of the entire protein. Interestingly, the membrane-pore-forming unit, comprising helices 5–9 (ref. 4) or 3–9 (ref. 2), is essentially identical to the globin-like domain (helices 4–9). In addition, recent spectroscopic results (J. H. Lakey *et al.*, manuscript submitted) show that this unit remains closely packed on membrane insertion rather than opening up like an umbrella as initially suggested<sup>2</sup>. Bacteria could have acquired this domain by adapting a gene fragment of some, as yet unidentified, protein with the globin fold.

A substantial fraction of known protein structures can be described in terms

of a relatively small set of folding motifs, such as helical bundles,  $\beta$ -barrels and the like. Our finding establishes the three-on-three helical sandwich of the globin fold as one such prototypic motif used by proteins in various functional contexts.

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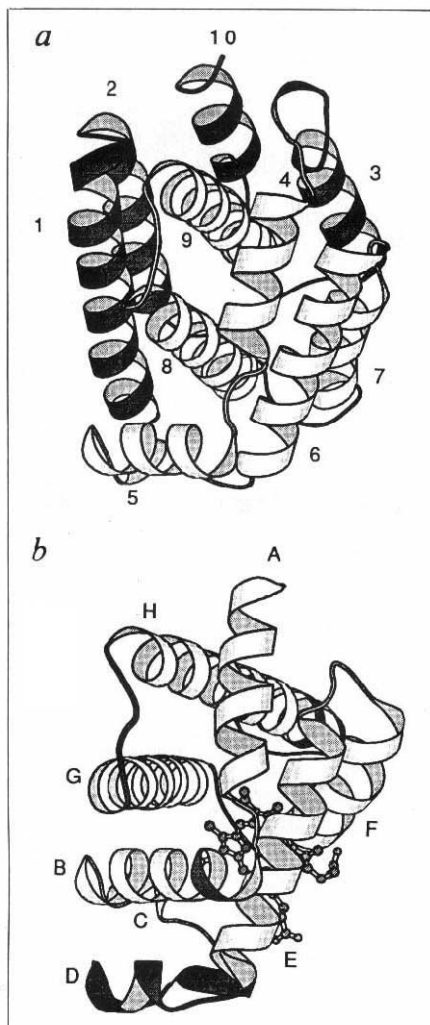
1. Holm, L. & Sander, C. *FEBS Lett.* **315**, 301–306 (1993).
2. Parker, M. W., Pattus, F., Tucker, A. D. & Tsernoglou, D. *Nature* **337**, 93–96 (1989).
3. Pastore, A. & Lesk, A. M. *Proteins* **8**, 133–155 (1990).
4. Liu, Q. R. *et al.* *Proteins* **1**, 218–229 (1986).
5. Kraulis, P. J. *J. appl. Cryst.* **24**, 946–950 (1991).

## HIV tropism

**SIR** — Nara *et al.* point out<sup>1</sup> that cell surface factors other than CD4 influence HIV-1 tropism *in vitro*, but it has been known for some years that HIV-1 isolates that vary in genetic composition replicate at different rates in transformed CD4<sup>+</sup> cell lines and in primary cells (reviewed in refs 2–4). All *in vitro* experimental systems, of course, require careful interpretation.

The underlying reasons for the apparent correlation between the sensitivity of HIV-1 isolates to neutralization by soluble CD4 (sCD4) and the ability of these isolates to replicate in transformed T cells are imperfectly understood. Nara *et al.* emphasize the effect of the target cell on the sCD4-sensitivity of HIV-1, but fail to note reports that document the importance of viral factors<sup>5–7</sup>. The data of Nara *et al.* are derived from studies using HIV-1 IIIB, which is a highly adapted virus, so it is potentially misleading to draw general inferences from studies with this virus. Cell line-adapted HIV isolates such as IIIB or RF are up to 1,000-fold more sensitive to sCD4 neutralization than primary viruses, irrespective of whether peripheral blood mononuclear cells or cell lines are used to monitor their replication<sup>5–7</sup>.

HIV-1 did not evolve to resist neutralization by sCD4, but it may well have



**FIG. 1** Ribbon diagrams<sup>5</sup> of *a*, the carboxy-terminal fragment of colicin A (Protein Data Bank entry 1COL) and *b*, myoglobin (Protein Data Bank entry 1PMB). The common core has lighter shading. The equivalent  $\alpha$ -helices in colicin A and globins, respectively, are helix 4 = A, 5 = B, 6 = E, 7 = F, 8 = G and 9 = H. The ribbon diagrams highlight the similar overall fold of the common core, in spite of small shifts in the relative orientations of some of the helix pairs. The structural alignment method<sup>1</sup> picks up 112 structurally equivalent residues between colicin A and myoglobin, which in rigid-body superimposition give a root mean square deviation of  $\alpha$  positions of 3.2 Å. The equivalent segments follow (1COL/1PMB): A73–A89/A4–A20, A90–A109/A24–A43, A110–A128/A61–A79, A129–A135/A81–A87, A136–A145/A89–A98, A149–A166/A99–A116, A167–A187/A123–A143.

done so to counter neutralizing antibodies in general, and those directed at its CD4-binding site in particular. These antibodies are highly prevalent in HIV-positive human serum<sup>8</sup>. The requirement of HIV-1 for protection against neutralization may be lost when the virus is grown in tissue culture and more rapidly growing strains are selected. Coincidentally or otherwise, such strains may be more sensitive to neutralization in general, and to neutralization by sCD4 in particular. The neutralization resistance of primary viruses might have dire consequences for the chances of successful therapeutic intervention by the administration of neutralizing antibodies.

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**SIR** — Nara *et al.*<sup>1</sup> noticed that in studies with chimaeric viruses, designed to map viral determinants for macrophage- and T-cell-line tropism, replication of all the variants in peripheral blood mononuclear cells (PBMC) was ignored. In fact it is well known that PBMC are the susceptible target cells for all variants of HIV-1<sup>9–11</sup>. Nara *et al.* suggest that “viral envelope elements that otherwise abolish infectivity in cell lines are tolerated in PBMC, suggesting fundamental differences of viral entry at the cellular level for these cells”. It is the permissiveness of PBMC that they call ‘enhanced permissivity’ or ‘universal tropism’. Rather than restrictive viral determinants, they now assume active cellular repression of the entry process.

Is this a matter of semantics or is it a real change in paradigm and, if so, what is to be gained? Apart from the fact that target cells hardly affect the neutralizing capacity of sCD4 (ref. 7), it is unclear how this concept of universal tropism contributes to the unravelling of the molecular mechanisms involved. Moreover, it has been demonstrated that also in PBMC differences in *env* gene-related biological properties exist, indicating that PBMC are not fundamentally different from other cell-types in this respect<sup>2,9</sup>. The prevailing interpretation is that monocytes and T-cell lines carry distinct secondary receptors that discriminate for monocyto-tropic and T-cell-line tropic isolates. The susceptibility of PBMC for all virus isolates therefore might be due to the expression of both these secondary receptors on each primary cell or to the expression of these receptors on different cells present in the mixture that constitutes the PBMC population. The fact that macrophage- and T-cell-line tropism can be recipro-

cally exchanged by *env* gene fragments suggests that this virus element can distinguish between the additional receptor on the surface of these two cell types<sup>12,13</sup>. This knowledge of the viral *env* gene-encoded interaction site(s) will aid in the identification of putative additional receptors. Moreover, for this purpose cell types that express distinct additional receptors are required rather than PBMC, which will not allow discrimination of possible receptors.

Although PBMC should be used in primary isolation studies to obtain the complete spectrum of virus isolates, we fail to see what the impact of the new concept of ‘universal tropism’ will be on strategies for unravelling cell tropism of HIV-1 variants.

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1. Nara, P. *et al.* *Nature* **360**, 215–216 (1992).
2. Fenyö, E. M., Albert, J. & Åsjo, B. *AIDS* **3**, S5–S12 (1989).
3. Cheng-Mayer, C. *AIDS* **4**, S49–S56 (1990).
4. Moore, J. P. & Nara, P. *AIDS* **5**, S21–S33 (1991).
5. Daar, E. S., Li, X. L., Moudgil, T. & Ho, D. D. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6574–6578 (1990).
6. Turner, S. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **89**, 1335–1339 (1992).
7. Moore, J. P. *et al.* *J. Virol.* **66**, 235–243 (1992).
8. Moore, J. P. & Ho, D. D. *J. Virol.* (in the press).
9. Tersmette, M. *et al.* *J. Virol.* **63**, 2118–2125 (1989).
10. Åsjo, B. *et al.* *Lancet* **ii**, 660–662 (1986).
11. Schultemaker, H. *et al.* *J. Virol.* **65**, 356–363 (1991).
12. O'Brien, W. A. *et al.* *Nature* **348**, 69–73 (1990).
13. Shioda, T., Levy, J. A. & Cheng-Mayer, C. *Nature* **349**, 167–169 (1991).

## Neurotransmitter role for ATP?

**SIR** — It would be unfortunate if the idea that ATP is commonly a cotransmitter at cholinergic synapses<sup>1</sup> entered the textbooks, as there is so far no evidence for this at any cholinergic synapse. Neither the synapses studied in slices containing the habenula<sup>2</sup>, nor those in cultured sympathetic coeliac ganglia<sup>3</sup> are cholinergic. All fast excitatory preganglionic synaptic events in neurons of intact guinea pig coeliac ganglia are rapidly and reversibly abolished by tubocurarine or hexamethonium<sup>4</sup>, as in all autonomic ganglion cells studied so far. The lack of effect of the P<sub>2x</sub> purinoceptor antagonist suramin at intact ganglionic synapses has recently been confirmed<sup>5</sup>. The purinergic ‘autapses’ which formed between cultured coeliac neurons<sup>3</sup> were presumably made by the terminals of those noradrenergic neurons<sup>6</sup> which normally end on blood vessels within the gastrointestinal tract. Blockade of vaso-

constriction and of excitatory junction potentials in these arterial vessels by suramin<sup>5</sup> are taken to indicate that ATP is released from perivascular endings.

We are unaware of any direct evidence that ATP and acetylcholine are co-released from any one type of peripheral nerve terminal. Stimulation of the innervation of cholinergically innervated visceral organs such as the bladder may produce atropine-resistant responses which are blocked by purinoceptor antagonists<sup>7</sup>, but there is no reason to conclude that these necessarily arise from cholinergic terminals. The neurochemistry of non-adrenergic postganglionic neurons innervating the bladder is diverse<sup>8,9</sup>, and at least two types of non-adrenergic neuron may innervate the muscle<sup>10</sup>. As there is currently no marker to distinguish cholinergic from non-cholinergic neurons in the peripheral nervous system, the possibility that two sets of terminals are present cannot be excluded.

On the other hand, the results reported by Evans *et al.*<sup>3</sup> are unexpected. The abolition by cholinergic antagonists of fast excitatory synaptic events between cultured rat superior cervical ganglion cells<sup>11</sup> is the basis for our current understanding of the plasticity of sympathetic neurons in the transmitter phenotype they express under different environmental influences<sup>12</sup>. Whether the synapses that Evans *et al.* studied were made by the terminals of noradrenergic vasoconstrictor neurons which had retained their original purinergic properties, or whether the terminals had been converted to purinergic ones by environmental influences *in vitro*, remains to be confirmed. One wonders whether, after a longer period in culture, the autapses in the coeliac neurons would have become cholinergic, as in the experiments of O'Lague *et al.*<sup>11</sup>.

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1. Benham, C. D. *Nature* **359**, 103–104 (1992).
2. Edwards, F. A., Gibb, A. J. & Colquhoun, D. *Nature* **359**, 144–147 (1992).
3. Evans, R. J., Derkach, V. & Surprenant, A. *Nature* **357**, 503–505 (1992).
4. McLachlan, E. M. & Meckler, R. L. *J. Physiol., Lond.* **415**, 109–129 (1989).
5. Evans, R. J. & Surprenant, A. *Br. J. Pharmac.* **106**, 242–249 (1992).
6. Macrae, I. M., Furness, J. B. & Costa, M. *Cell Tiss. Res.* **244**, 173–180 (1986).
7. Creed, K. E., Ito, Y. & Katsuyama, H. *Am. J. Physiol.* **261**, C271–C277 (1991).
8. Crowe, R., Haven, A. J. & Burnstock, G. *J. auton. Nerv. Syst.* **15**, 319–339 (1986).
9. Keast, J. R. & de Groat, W. C. *J. comp. Neurol.* **288**, 387–400 (1989).
10. James, S. & Burnstock, G. *Regul. Peptides* **28**, 177–188 (1990).
11. O'Lague, P. H., H. *et al.* *Cold Spring Harbour Symp. quant. Biol.* **50**, 399–407 (1976).
12. Landis, S. C. in *Autonomic Ganglia* (ed. Elfvin, L.-G.) 453–473 (Wiley, Chichester, 1983).



# Prophet for the Earth

Stephen Jay Gould

**The Diversity of Life.** By Edward O. Wilson. *Belknap (Harvard University Press)/Allen Lane: 1992. Pp. 424. \$29.95, £22.50.*

WE meet, and need, leadership in many guises. Sometimes we crave statesmen or generals; but other moments require prophets. Jeremiah began his lamentations over the captivity of Jerusalem with a powerful image of emptiness: "How doth the city sit solitary, that was full of people. . . . She that was great among the nations . . . how is she become tributary." He then switched to a biological metaphor (Lamentations 1:6): "Her princes are become like harts that find no pasture; and they are gone without strength before the pursuer". If, as Pogo said, the enemy is us, then we are the huntsmen, the destroyers of habitat, the blight upon former plenitude.

We all know about dodos and great auks as abstractions or stuffed specimens in museums. But for most people most of the time, the wave of extinctions unleashed by human depredation of our earthly environment ranks as something distant from immediate locales and concerns (however global the ultimate threat). Therefore we need prophets to shake the souls and grab the attention of those who have eyes but see not. *The Diversity of Life* is a deft and thoroughly successful mixture of information and prophecy.

Wilson has composed his text in three parts: first, on natural extinctions (and recoveries) at scales rising from the local and historical (Krakatoa in 1883) to geological and global (the five great mass dyings of our palaeontological record); second, on the evolutionary construction and richness of biodiversity; and third, on human disruptions, malfeasances and (if we are both lucky and smart) potential reversals and solutions. The connecting thread is the familiar theme that our current geological micromoment of anthropogenic carnage ranks as a full-blown sixth episode of mass extinction, not as one of those pervasive blips that mark planetary business as usual — in other words, a rare and portentous punctuation, not a part of the permeation. (On this connecting theme, and with apologies for a petty comment from my own parish, may I request that subsequent printings of this book — a great and fit work destined to survive and thrive well into the next millennium — correct the numerous palaeontological errors scattered throughout: foraminiferal tests are generally calcareous, not siliceous; Pan-

gaea did not congeal until the late Palaeozoic, and therefore did not exist during the Ordovician and Devonian mass dyings.)

But what combination of influences can recast a celebrated academician into the unfamiliar role (in our church) of effective prophet. Above all, of course, one must have a cause — and what purpose could be more noble than speaking for (the literal meaning of prophet, by the way) the multitude whose voices do not broadcast on human wavelengths — the 1.5 million or so described species that stand for an unknown bounty ranging up to 100 million or more? Prophets must speak for uncompromised principles, though we all know that practical solutions will fall short. Wilson's credo is ringing and simple: fight to save all taxa. He writes:

In democratic societies people may think that their government is bound by an ecological version of the Hippocratic oath, to take no action that knowingly endangers biodiversity. But that is not enough. The commitment must be much deeper — to let no species knowingly die, to take all reasonable action to protect every species and race in perpetuity.

Putting such a credo into action requires great powers of persuasion in two different spheres. First, knowledge: few nonspecialists appreciate either the taxonomic richness of nature or the magnitude of current loss. (Wilson estimates that "a fifth or more of species of plants and animals could vanish or be doomed to early extinction by the year 2020 unless better efforts are made to save them".)

Moreover, we who study biodiversity face the correlated problem of inadequate respect for our efforts. We are too often seen as superfluous accountants — mere listers and arrangers, scribes in a catalogue of particulars containing no messages about general theories of natural order. And yet, the historical data of unique items in contingent sequences are as intrinsically valuable and theory-laden as the most rigidly predictable and repeatable behaviour of molecules or planets in deterministic systems. Data of our sort, after all, fuelled Darwin's documentation of one of the great generalities in our intellectual history: evolution by natural selection.

The number of trained taxonomists has been so depleted that the diversity of many groups of major economic import-

ance cannot be studied at all. (Wilson reports, for example, that only one scientist in North America studies the classification of oribatid mites on a full-time basis — although these soil-dwelling arthropods are ubiquitous consumers of humus and fungal spores, and are "therefore key elements of land ecosystems almost everywhere".) Yet there is a way, if only we had the will (emerging from an understanding of importance); nature's richness need not stymie us. Wilson reports that, at the "cautious pace" of 10 species per year, 25,000 professional lifetimes of 40 years could lead to the description of 10 million species (a low but popular estimate of global biodiversity). Such a number still represents "less than 10 per cent of the current population of scientists active in the United States alone".

Second, empathy (the main thrust of prophecy): Jesus said that the truth shall make us free, but how can we transfer to the gut of feeling (and desire for action) what we may know in our heads? This problem is particularly difficult when most victims are not starving humans (at least not yet), or even furry pandas, but cockroaches, nematodes and mites.

The practical reasons for preserving unknown species (primarily agricultural and pharmacological) are well documented, and thoroughly discussed by Wilson. But I have always thought that a movement for salvation cannot succeed without a primary grounding in the better and broader arguments: ethical (what right has a tiny twig to expunge great limbs from its nurturing tree?) and aesthetic (the sheer joy of such limitless variety versus a depleted landscape of people, dogs, rats, pigeons and flies). But these moral and emotional arguments need both knowledge and love of nature, and how do we foster the latter in an urban society offering little direct exposure to most citizens?

We can take people to wilderness (so-called 'ecotourism'), though no general solution can emerge from this basically elitist strategy. We can rely upon film and other burgeoning media of our electronic revolution, but these vicarious strategies too often foster passivity. I am old-fashioned enough to believe in the primacy and efficacy of great writing (well-illustrated of course), and I thank all our predecessors for their legacy of eloquent support. I have often argued that the best popular writing about science falls into two traditions: the Galilean or rationalist that locates the thrill of nature in its intellectual puzzles (with Huxley, Haldane and Medawar as the great practitioners since Darwin's day) and the Franciscan or lyrical that can capture the visceral beauty — 'nature poetry', if you will, from St Francis of Assisi through W. H.

Hudson, Henri Fabre, and Loren Eiseley or John McPhee in our day. Wilson is a celebrated professor at an institution placed (however undeservedly) on a pinnacle of rarefied intellectual etheriality, but when he lets go (as he does here, and as I wish he would do more often), he is the finest Franciscan naturalist of our time. Wonderful writing! (I say this with some jealousy as the most committed of Galileans who, by personal limitation, can do no other. *Ne supra crepidam sutor judicaret.*)

Reviews of such praise must also disgorge some dyspepsia, and I am not without my criticisms. It is no secret that Wilson and I look at evolution quite differently (from joint residence within such an ample Darwinian estate), and I do deplore some unkind and unfair statements about punctuated equilibrium. But if there were ever a subject for beating the swords of professional dispute into ploughshares of joint action, then preservation of biodiversity is our common strength and shield. (Good Lord, we can even bring animal rights activists — and, dare I say it, even some creationists — under this banner; so why should such lesser disputes as adaptation and contingency, or human sociobiology and culturally driven flexibility, not share common cause?)

Still, I do wish that I could dissuade my colleague from his commitment to pervasive adaptation and progress (by showing him the incredible yet comprehensible strangeness of the fossil record viewed in detail). I confess that I do get cross — since he knows as well as I do that we live in the Age of Bacteria (as it was in the beginning, is now and ever shall be, until the world ends) — when I read, in his book, the old ladder spiel of superannuated textbooks: “They [the first land invertebrates] were followed by amphibians . . . and a burst of land vertebrates . . . to inaugurate the Age of Reptiles. Next came the Age of Mammals and finally the Age of Man.” This, after all, is the very enshrining of arrogance and exaggerated self-importance that we must avoid if we are ever to take fellowship with nature seriously — and thereby avert the biodiversity crisis.

I also cannot accept the too-facile argument that we are likely to prevail thanks to an innate “*biophilia*, the connections that human beings subconsciously seek with the rest of life. To biophilia may be added the idea of wilderness . . . . Wilderness is a metaphor of unlimited opportunity, rising from the tribal memory of a time when humanity spread across the world”.

How can this be? Why should these valued features be deeper, more innate, more definitive of our nature, than our rapacity? Was it any less natural to kill



**SAFFRON gatherers** — our knowledge of Aegean art and society owes much to the discovery of wall paintings at Akrotiri, Thera, in the excavations of 1967–1974 directed by the late Professor Spyridon Marinatos. The paintings have been remarkably preserved by the thick layer of ash that coated them in the volcanic eruption of 1628 bc. This detail is taken from *The Wall-Paintings of Thera* by Christos Doumas, a lavishly illustrated volume that shows all the frescos together for the first time. Thera Foundation, £39.

all the moas of New Zealand, all the mammoths of North America? Surely for each biophile in the United States, there are ten who would kill a deer for sheer sport, rather than for needed food; ten who will build the suburban shopping mall for each cry of “woodsman, spare that tree”.

We cannot win by appealing to an innate urge stronger than competing drives. Does Wilson really view these competing drives as mere cultural overlay, with biophilia and love of wilderness as primary biology yearning to emerge? Sorry, but such a view, to me, is romantic nonsense (and I use this word in the literal, not the pejorative, meaning). Here I do state my preference for rationalist Galilean solutions over pure Franciscan lyricism. The best way must be learned and studied, not simply freed from an internal reservoir.

We are a swirling congerie of biological potential for good and bad, preservation of biodiversity or global clear-cutting. The tendencies for good and right resolutions are within us, but so are the propensities for evil and short-sightedness. Let the spring of En Gedi be a symbol for the fount of goodness within us. Wilson recounts the story of his visit to this wondrous oasis of greenery and biodiversity in the harsh Judean

desert (and I could tell a similar tale of my own encounter). En Gedi is the Franciscan paradise of Solomon’s song: “My beloved is unto me as a cluster of camphire in the vineyards of En-gedi” (Song of Songs 1:14). And En Gedi is also the great Galilean symbol of moral restraint upon murder, for here David could have ambushed a defenceless Saul, yet desisted (1 Samuel, 23–24).

Wilson quotes the wonderful motto of the Senegalese conservationist Baba Dioum — and what better union of love and knowledge could we seek to unleash and empower the right side of our vast biological potentiality: “In the end, we will conserve only what we love, we will love only what we understand, we will understand only what we are taught.” This union of love and knowledge is called wisdom, and wisdom, like nature, is truly “a tree of life to them that lay hold upon her” (Proverbs 3:18). With a proper fusion of Galilean intelligence and Franciscan love, we may be able to honour E. O. Wilson as a prophet in his own country — which has become, in our truly ecumenical age, the whole world. □

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## Ancient roots

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**The Great Copernicus Chase and Other Adventures in Astronomical History.** By Owen Gingerich. Cambridge University Press: 1992. Pp. 304. \$19.95, \$29.95.

SCIENCE began with astronomy (or so it can be argued), when humankind first tried to codify the operations of the external world of nature. Repeatable observations may have sparked the idea of repeatable experiments; and the obvious start, the reliable recurrence of sunrise and sunset, could have led to awareness that the sunset direction changed from day to day, but then became near-constant at midsummer or midwinter. Why not plant two standing stones to mark this special sunset point? Would the setting Sun manage to creep to the same point next year? Yes, it did, and some bold theorist may have made the (scientific?) prediction that it would get there every year. Perhaps things did not happen like that, but much tangible evidence in durable stone may be trying to tell us that they did.

With the mystique of these ancient roots to help, the history of astronomy has an inherent fascination, which no one in recent years has communicated more effectively than Owen Gingerich. Now, between the covers of this book, 36 of his fluent and expert essays on astronomical history are reprinted: 26 were published in *Sky and Telescope* in the 1970s and 1980s, and ten elsewhere. The order is chronological, from the sky magic of the ancient Egyptians to the comet of 1965; the recipe is a clear, urbane and judicious style; and the result is a feast for astrophiles.

One nourishing item on the menu is the astronomy of Stonehenge. For Gingerich, Stonehenge is "not so much an ancient megalithic observatory as the monument to an earlier observatory". The first Stonehenge was an earthwork circle about 100 metres in diameter and 2 metres high, broken (about 2400 BC) by a single gap through which passes the line from the centre to the heel stone marking midsummer sunrise. After 300 years the smaller circle of heavy stones (Stonehenge 3) was set up to "fossilize" this successful observatory. The first Stonehenge was more accurate, as it had longer sightlines and smaller stones, though Gingerich is sceptical of the idea that it was used for calculating eclipses. He takes great care to explain the astronomical alignments, and produces paper cutouts to be stuck on a cola tin to help in visualizing the gyrations of the Earth and the apparent motion of the Sun.

The Great Copernicus Chase in the

title refers to Gingerich's obsessive pursuit of first (and second) editions of Copernicus's epoch-making *De Revolutionibus Orbium Coelestium*, first published in 1543. The chase has taken him all over Europe (and elsewhere), and some good stories are woven into the tapestry of finicky scholarship. So far he has found 245 copies of the first edition. They differ, if at all, in their handwritten notes, and the identification of the hands keeps his ingenuity at full stretch.

Ingenuity is the watchword too in several other essays. When Gingerich tells us about the discovery of the two small satellites of Mars in 1877, he also faces the question, "How did Jonathan Swift manage to specify their orbits so well in 1726, in *Gulliver's Travels*?" Ingeniously, Gingerich notes that Swift predicted the radii of the satellites' circular orbits as three and five times the planet's diameter: this is the same as for Io and Europa, satellites of Jupiter. Whether Swift knew this is questionable, but if he did his clairvoyance fades into opportunistic unoriginality.

Maybe there was "nothing new under the Sun", or at most not much, in the era of Islamic dominance; but at least astronomy remained alive and well between the eighth and fourteenth centuries. Gingerich's long essay on Islam is notable for his careful explanation of the astrolabe and its functions. Two other essays are on astrolabes: one describes a particular specimen, the other is a cautionary guide to fake astrolabes.

A fake of wider interest is the "great comet that never was" of 1857. In Paris particularly, this mythical monster was widely expected to collide with the Earth on 13 June. Apparently this idea originated in an astrologer's forecast, which was based on a wrong interpretation of a genuine prediction that a previous comet would reappear in 1848, though this prediction itself proved to be wrong. The non-appearance of the non-comet only heightened the tension, and comet-proof clothing was designed, rather as in 1979, when anti-Skylab steel helmets were put on sale.

The essays are beguiling and well-written. Reading them *en masse* does, however, expose some weaknesses. Often the narrative flows along with panache but then seems to peter out in a lame and tentative conclusion. Possibly this has happened because *Sky and Telescope*, like all successful journals, must take care not to offend too many of its readers. With this proviso, the essays are well worth reading (or re-reading), and the book will bring them the wider audience they deserve. □

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## Light exercise

R. McNeill Alexander

**Advanced Materials for Sports Equipment.** By K. E. Easterling. Chapman and Hall: 1992. Pp. 127. £17.50 (pbk).

MANY people are prepared to spend a lot of money on equipment for sport, even if they play only for recreation and at a modest level. There are plenty of manufacturers competing to satisfy the demand for equipment that may boost our egos by enabling us to perform just a little better, even if it has to be expensive. Much of the equipment they produce is built to elaborate design, from unconventional materials. Some running-shoe soles have pockets of gas encapsulated in polyurethane foam to make them springy; and some bicycle frames are made of silicon carbide fibres embedded in an alloy of copper and aluminium. What good does all this sophistication do? Why should we pay the extra price?

In a few cases, the benefits are clear. The world record for the pole vault increased dramatically from around 4.75 metres to more than 6 m as bamboo poles were replaced by aluminium ones and then by poles made of carbon fibre-reinforced epoxy resin. Conventional steel bicycle frames weigh 2 to 3 kilograms but carbon fibre frames can weigh as little as 1.2 kg. In many other cases the benefits of the new materials are less evident and may even be imaginary.

Easterling, an engineer, sets out to explain the new materials. His book is designed for intelligent sports enthusiasts with little, if any, scientific knowledge; but unfortunately he seems to feel unable to explain basic science to them.

He tells us that cracks spread less easily through composite materials such as fibreglass than through conventional materials, but explains why in only a superficial way. He tells us that panels of sandwich construction (for example, a metal honeycomb between solid plates) combine stiffness with lightness, but omits the rudiments of bending theory that explain why. He tells us how a racket responds when a ball strikes the sweet spot but does not explain the underlying principle. Nonscientists who want real understanding will be disappointed, and scientists will be annoyed by some sloppy use of technical terms. The book will nevertheless be welcomed by everyone who wants to know about the materials hidden below the glossy finish of modern equipment. □

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# Treatise on man

Ian Tattersall

**The Cambridge Encyclopedia of Human Evolution.** Edited by Steve Jones, Robert D. Martin and David R. Pilbeam. Cambridge University Press: 1992. Pp. 506. £60, \$95.

WHEN any major reference work — especially one that has involved the coordination of more than 70 contributors — has spent as long in gestation as this one, two questions immediately spring to mind when it finally appears: “Was it worth the wait?” and “Can it possibly be up to date?”. Happily, perusal of *The Cambridge Encyclopedia of Human Evolution* suggests that the answer to both questions is “yes”. But it also evokes a less obvious question: “Is this work properly described as an encyclopaedia?”. Here the answer is probably “no”, certainly if we compare it with the majority of other encyclopaedias in the Cambridge series, or most especially with its obvious competitor by size and title, the *Encyclopedia of Human Evolution and Prehistory*, published in 1988 by Garland (in which this reviewer, as a co-editor, must declare an interest — for a review see *Nature* 335, 598; 1988). The present volume is not an encyclopaedia in the traditional sense, with a large number of entries, alphabetically arranged. Rather, it is better described as a treatise, with about 80 short chapters, some by several authors, grouped into ten main subject areas.

Whether it is adequately described as a treatise on human evolution is yet another question. The editors clearly adhere to the conceit that the study of human evolution legitimately embraces virtually all the principal areas of human biology, stone-age archaeology and primatology; and this shows in the book’s extremely broad coverage, which extends to embrace most of the field of physical anthropology. The price paid for this remarkable comprehensiveness is a loss of focus on human evolution proper, which on a liberal estimate accounts for less than half the volume’s length.

Such quibbles aside, however, the work provides a feast of information for anyone interested in the history of our species and in the allied disciplines that have contributed to our understanding of this history. The volume opens with a consideration of evolution (long on molecular genetics and short on macroevolution), classification, and the diversity of living primates and how to conserve it. The next sections take up a variety of topics in primate biology,

behaviour and social organization, with a particular look at the brain and its role in communication and language. There follows a group of brief contributions on fossils, chronometric dating and taphonomy, and on the influences of climate change and continental movement on evolution; this as a prelude to a short section (only a little over 50 pages) dealing with the primate and human fossil records. The next 80 pages are devoted to a diversity of genetic topics, ranging from the basics of genetics to molecular methods for deducing evolutionary sequences. There’s lots of good information here, but the relationship between genetics and evolution remains vague and is mired in a microevolutionary perspective: one searches in vain for any discussion of speciation or of macroevolutionary mechanisms.

The book then proceeds to consider a variety of topics in the reconstruction of early human behaviour and ecology, principally on archaeological evidence and extending up to the domestication of animals and plants. Again, there’s plenty of good stuff, but the ten chapters in this section don’t quite add up to a coherent account of what the archaeological record can tell us about our past. Following this we arrive at an account of the dispersal and biology of modern human populations, principally from a genetic and demographic perspective, as a prelude to the by now almost obligatory consideration of our species’ evolutionary future. Once more the perspective is microevolutionary, but from an entirely different vantage point I would unhesitatingly fall in with the view articulated here that innovation in human evolution is virtually at an end. A final section of appendices provides potted biographies of historical worthies, a Cenozoic time-scale, a world map of principal human fossil sites and a glossary of terms.

No work with over 70 authors could ever hope to be a completely comprehensive, consistent and seamless account of a wide-ranging area of science, and it would be pointless to criticize this remarkable volume for failing on any of these scores. It is much more appropriate to focus on the extraordinary variety of information that has been packed between its covers. Nobody will be able to browse through this book without discovering something new; a fairly comprehensive index (and a little cross-referencing in the text) will in some measure compensate for its structural dissimilarity to traditional encyclopaedias; it is bang up to date (how often has a book arrived on your desk that contained a reference to an article published only three months earlier?); and if its view of evolution is a trifle old-fashioned, it does no more than reflect the state of the art in its field. If

(whether student or professional) you’re interested in human evolution, you’ll want to have this volume handy. □

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## Goodbye to Gutenberg?

Graham Dunn

**Light Microscopy: An Electronic Textbook.** By D. J. Rawlins. Bios Scientific: 1992. £52.88 (UK and EC), £45, \$90 (elsewhere) (single licence); £158.63 (UK and EC), \$270 (elsewhere) (department licence).

ENTER the world’s first electronic textbook on light microscopy. Will the two 3½-inch floppy disks held in my trembling fingers herald a new age in which printing will become obsolete and teachers redundant? My disillusionment was slow but profound. Slow because this was no ordinary book that could be scanned simply by flicking pages, and profound because it might as well have been. Or at least it might have made a modest pamphlet: I estimate that the total textual content of “over 200 pages of information” would fit comfortably on six pages of *Nature*. Certainly it would then be easier to read but it would be harder to justify the “in-depth coverage” proclaimed by the advertising leaflet, or for that matter, the price.

To be fair though, the bulk of the information contained in the electronic textbook is devoted to producing images, not text, and a few of these approach the quality of colour photographs. But even these would be better printed on glossy paper, because no animations or computational techniques are applied to them. Computers do have an important role as teaching aids but only if their ability to perform computations and selectively guide the user’s access to a large body of information is exploited. Little of this kind is on offer here and I feel that an exciting opportunity has been missed. Teachers of light microscopy may breathe again — for a while. □

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■ Also just published by Bios Scientific is D. J. Rawlins’s *Light Microscopy*, a “complementary book for first-time users”. Pp. 156. £14.50, \$29.

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# Inositol trisphosphate and calcium signalling

Michael J. Berridge

**Inositol trisphosphate is a second messenger that controls many cellular processes by generating internal calcium signals. It operates through receptors whose molecular and physiological properties closely resemble the calcium-mobilizing ryanodine receptors of muscle. This family of intracellular calcium channels displays the regenerative process of calcium-induced calcium release responsible for the complex spatiotemporal patterns of calcium waves and oscillations. Such a dynamic signalling pathway controls many cellular processes, including fertilization, cell growth, transformation, secretion, smooth muscle contraction, sensory perception and neuronal signalling.**

A DECADE has passed since the first report of a second messenger function for inositol(1,4,5)-trisphosphate ( $\text{InsP}_3$ )<sup>1</sup>. In that time we have found out how it is produced, how it is metabolized and, now that its receptor has been cloned, we are beginning to understand how it acts to release calcium<sup>2-6</sup>. The recent disclosure that Lowe's oculocerebrorenal syndrome is caused by a defect in a gene that encodes one of the enzymes that metabolizes  $\text{InsP}_3$  (ref. 7) will ensure a continuing interest in this messenger pathway well into the next decade.

It is through its regulation of intracellular calcium that  $\text{InsP}_3$  functions to regulate so many cellular processes<sup>2,3,5,6</sup>. In response to many stimuli (such as neurotransmitters, hormones and growth factors) both  $\text{InsP}_3$  and diacylglycerol (DAG) are formed by the hydrolysis of an inositol lipid precursor stored in the plasma membrane. The  $\text{InsP}_3$  released into the cytoplasm mobilizes calcium from internal stores, whereas DAG activates protein kinase C (ref. 8). This bifurcating messenger system operates throughout the life of a typical cell, beginning with gametogenesis, fertilization, cell proliferation and early development and continuing through differentiation to perform very precise control functions in a whole variety of specialized cells in both animals and plants. Before describing its role in some specific cellular processes, I shall summarize how  $\text{InsP}_3$  is released from the membrane and how it acts to generate calcium signals.

## Signalling pathways and molecular heterogeneity

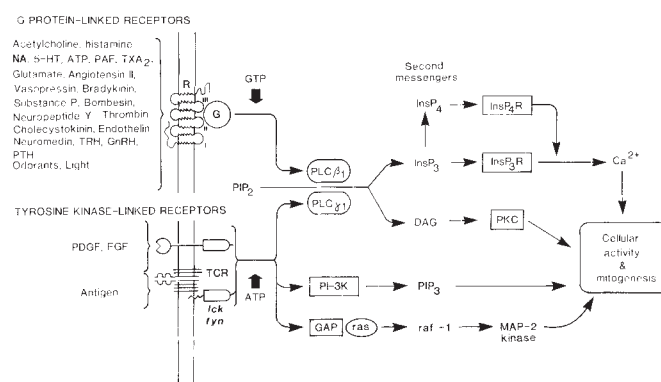
External signals arriving at the cell engage surface receptors to initiate signalling pathways whereby information flows from one component to the next until the final effector system is activated. The formation of  $\text{InsP}_3$  is the focal point for two major pathways, one initiated by a family of G protein-linked receptors and the other by receptors linked by tyrosine kinases either directly or indirectly (Fig. 1). These separate receptor mechanisms are coupled to energy-requiring (GTP or ATP) transducing mechanisms which activate phospholipase C (PLC) to hydrolyse the lipid precursor phosphatidylinositol 4,5-bisphosphate ( $\text{PtdIns}(4,5)\text{P}_2$ ) to give both DAG and  $\text{InsP}_3$ . The latter then binds to an  $\text{InsP}_3$  receptor ( $\text{IP}_3\text{R}$ ) to mobilize stored calcium and to promote an influx of external calcium, perhaps working in conjunction with  $\text{InsP}_4$  (refs 9, 10).

What is not apparent from Fig. 1 is the rich diversity of the individual components within these signalling pathways. Classical pharmacology prepared us for receptor diversity in that each of the stimuli listed in Fig. 1 acts through a separate receptor. What came as a surprise, however, was the extensive heterogeneity of the downstream elements, such as the G proteins (Box 1), PLC (Box 2),  $\text{IP}_3\text{R}$  (Box 3) and PKC (ref. 8). The full extent of this diversity is apparent in *Drosophila* photoreceptors where the transducing elements rhodopsin,  $\text{DG}_q$  and  $\text{norpA}$  (Box 1) are present primarily in the eye. Why did this diversity arise? We can only surmise that a few basic signalling systems emerged early in evolution and were then modified in

subtle ways to meet the unique signalling requirements of different cells. In order to detect brief flashes of light, photoreceptors face very different problems from liver cells responding to slower changes in circulating hormone levels. As cells differentiate to perform separate functions, they select out specific components from a diverse signalling repertoire to construct personalized messenger pathways precisely adapted to suit their particular requirements. Despite all this molecular heterogeneity, the common theme running through all these variations is that external signals use the  $\text{InsP}_3$ /calcium and DAG/PKC pathways to regulate a wide range of cellular activities.

## G protein-linked receptors

The membrane-transducing unit controlled by G protein-linked receptors has three main components—the activated receptor communicates through a G protein to stimulate PLC (Fig. 1 and Box 1). Most of the G protein-linked receptors identified so far are characterized by having seven membrane-spanning domains connected by extracellular and intracellular loops (Box 1). The transmembrane columns interact with each other to form a pocket where the agonist binds to induce the conformation responsible for activating the G protein, that is, the next component of the signalling pathway. This G protein family can be divided into two, depending on whether or not they are sensitive



**FIG. 1** Summary of the two major receptor-mediated pathways for stimulating the formation of inositol trisphosphate ( $\text{InsP}_3$ ) and diacylglycerol (DAG). Many agonists bind to 7-membrane-spanning receptors (R), which use a GTP-binding protein (G) to activate phospholipase C- $\beta_1$  (PLC- $\beta_1$ ), whereas PLC- $\gamma_1$  is stimulated by the tyrosine kinase-linked receptors (see Box 2). The latter activate other effectors such as the phosphatidylinositol 3-OH kinase (PI-3K), which generates the putative lipid messenger phosphatidylinositol (3,4,5)-trisphosphate ( $\text{PIP}_3$ ) and the GTPase-activating protein (GAP) that regulates *ras*.  $\text{InsP}_3\text{R}$ ,  $\text{InsP}_3$  receptor; PKC, protein kinase C; NA, noradrenaline; 5-HT, 5-hydroxytryptamine; PAF, platelet-activating factor;  $\text{TXA}_2$ , thromboxane  $\text{A}_2$ ; TRH, thyrotropin-releasing hormone; GnRH, gonadotrophin-releasing hormone; PTH, parathyroid hormone.

to pertussis toxin. The second and third cytoplasmic loops of the receptor (see Box 1 for details) has an essential role in activating a member of the heterotrimeric G-protein family<sup>17</sup> responsible for stimulating separate members of the PLC family (Box 1)<sup>18,19</sup>.

## Tyrosine kinase-linked receptors

The other pathway responsible for stimulating the release of  $\text{InsP}_3$  begins with tyrosine kinase receptors, which relay information through a direct interaction between the receptor and the  $\gamma$ -form of PLC (Fig. 1 and Box 2). As for the G protein-linked receptors, signal transduction through tyrosine kinase receptors is an energy-requiring process because ATP is consumed not only as the two receptors interact (autophosphorylation), but also during the subsequent phosphorylation of PLC- $\gamma_1$  (Box 2). Growth factors such as platelet-derived (PDGF) and epidermal (EGF) growth factors act by bringing two receptors together, which enables their cytoplasmic kinase domains to phosphorylate each other on tyrosine residues to create docking sites to bind PLC- $\gamma_1$ . In unstimulated cells, PLC- $\gamma_1$  is largely cytosolic but translocates to the membrane as its SH2 domain binds to the activated receptor (Box 2). This association has two important consequences for the activation of PLC- $\gamma_1$ , it is phosphorylated by the receptor on specific tyrosine residues and its membrane translocation brings it into contact with its substrate  $\text{PtdIns}(4,5)\text{P}_2$ . A similar activation process is responsible for stimulating PLC- $\gamma_1$  following crosslinking of IgM receptors in B lymphocytes or activation of the T-cell antigen receptor (TCR)-CD3 receptor complex of T cells. These lymphocyte receptors lack tyrosine kinase activity but they recruit members of the *src* family such as *fyn* and *lck* (Fig. 1).

The dynamics of different transducing mechanisms have been compared in NIH 3T3 cells, which carry both a G protein-linked receptor (bradykinin) and a tyrosine kinase-linked receptor (PDGF)<sup>24</sup>. When compared to bradykinin, the PDGF-induced formation of  $\text{InsP}_3$  was much slower, and the resulting calcium response was not only smaller but had a much longer latency.

Following transfection with a complementary DNA for PLC- $\gamma_1$ , the effects of bradykinin were unchanged, whereas the PDGF-induced responses were markedly enhanced; the rate of  $\text{InsP}_3$  formation was increased, resulting in a larger calcium signal with a reduced latency.

## $\text{InsP}_3$ and ryanodine receptors

$\text{InsP}_3$  and ryanodine receptors (RYRs) represent the two principal intracellular calcium channels responsible for mobilizing stored calcium. These two receptors will be considered together because they share considerable structural and functional homologies<sup>5,25</sup>. A family of  $\text{IP}_3\text{Rs}$  has now been identified (see Box 3)<sup>26,27</sup> with molecular diversity arising from both alternative splicing and the existence of separate genes<sup>28,29</sup>. The  $\text{IP}_3\text{R}$  contains typical membrane-spanning domains in the C-terminal region which anchor the protein in the membrane with four of the subunits combining to form the functional  $\text{InsP}_3$ -sensitive calcium channel (Fig. 2c). The large N-terminal domain lies free in the cytoplasm with the  $\text{InsP}_3$  binding site located at its end, a long way away from the channel-forming C-terminal region<sup>30</sup>. Upon binding  $\text{InsP}_3$ , the receptor undergoes a large conformational change which is perhaps related to the coupling process leading to channel opening<sup>30</sup>.

The other major intracellular calcium channel, the RYR, has three family members (Box 3). The RYRs identified in neurons<sup>41</sup> and sea urchin eggs<sup>42</sup> seem to resemble the cardiac RYR2. Some cells respond to transforming growth factor (TGF)- $\beta$  by expressing a much smaller RYR3 (ref. 34). Cells carrying RYR3 were insensitive to caffeine and ryanodine but the latter did abolish the effects of bradykinin, suggesting that RYR3 and  $\text{IP}_3\text{R}$  are co-localized. Like the  $\text{IP}_3\text{R}$ , RYRs exist as tetramers, with the C-terminal regions co-operating to form the calcium channel, and the large N-terminal regions forming bulbous heads that project into the cytosol (Fig. 2a and b). The two ways in which these RYRs respond to membrane depolarization is illustrated in Fig. 2. The plant alkaloid ryanodine opens the channels at low (nanomolar) concentrations but closes them at higher doses

### BOX 1 Signal transduction through G protein-linked receptors

#### Receptor-G protein-phospholipase C transduction unit

THE on-reaction begins when the agonist induces a conformational change in the receptor which is transmitted to the G protein loops II and III. The heterotrimeric G proteins dissociate into  $G_\alpha$  and  $G_{\beta\gamma}$  subunits, both of which can activate different PLC isozymes (see Box 2). The  $G_\alpha$  subunits exchange GDP for GTP before interacting with P and G sites on the C-terminal region of PLC- $\beta_1$  (ref. 11; see Box 2). There is growing evidence that the  $G_{\beta\gamma}$  subunit may also play a role<sup>12,13</sup>, particularly to activate PLC- $\beta_2$  (ref. 13). This membrane-transducing unit can be activated experimentally by injecting cells with GTP- $\gamma\text{S}$  or by treating them with fluoride. The venom mastoparan can also activate by mimicking the molecular interaction operating between the receptor and the G protein.

#### Phospholipase C functions as a GTPase-activating protein

During the off-reaction, GTP is hydrolysed to GDP by the GTPase associated with the  $\alpha$ -subunit which then re-combines with  $\beta\gamma$  to form an inactive complex.  $G_{\alpha q}$  has a very low intrinsic GTPase activity which is greatly

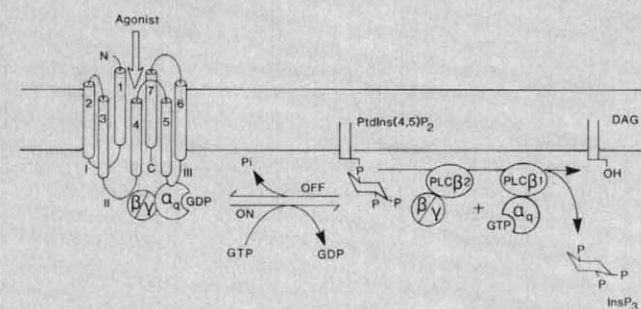
enhanced following its interaction with PLC- $\beta_1$ , suggesting that the latter functions like the GTPase-activating protein (GAP) which associates with the proto-oncogene p21<sup>ras</sup> (ref. 14). This finding that the effector molecule PLC- $\beta_1$  functions like GAP increases the likelihood that the latter might mediate the effects of p21<sup>ras</sup>.

| Receptors                   | G protein subunit                            | PLC            |
|-----------------------------|----------------------------------------------|----------------|
| Pertussis toxin-sensitive   |                                              |                |
| Glutamatergic (mGlu)        | $G_{\alpha o}$                               | ?              |
| Pertussis toxin-insensitive |                                              |                |
| Rhodopsin (fly)             | $DG_{\alpha q}$                              | norpA          |
| Muscarinic (M1, M3 and M5)  | $G_{\alpha q}, G_{\alpha 11}, G_{\alpha 14}$ | PLC- $\beta_1$ |
| Serotonergic (5-HT1c)       | $G_{\alpha 16}$                              | PLC- $\beta_2$ |
| Adrenergic ( $\alpha 1B$ )  | $G_{\beta\gamma}$                            | PLC- $\beta_2$ |

See Fig. 1 for further examples of pertussis toxin-insensitive receptors.

#### The role of cytoplasmic loops in coupling receptors to G proteins

Mutations in the third cytoplasmic loop of the adrenergic-1B receptor resulted in a constitutive activation of PLC, presumably by mimicking the active conformation normally induced by the agonist<sup>15</sup>. Substitution of alanine at position 293 (located in loop III close to transmembrane domain 6) with any of the 19 possible alternative amino acids all resulted in a constitutive increase in PLC activity. The wild-type receptor therefore has evolved a loop structure which gives the lowest basal activity. Transfection of cells with mutant receptors showing the highest constitutive activity results in transformation and tumorigenesis<sup>16</sup>.





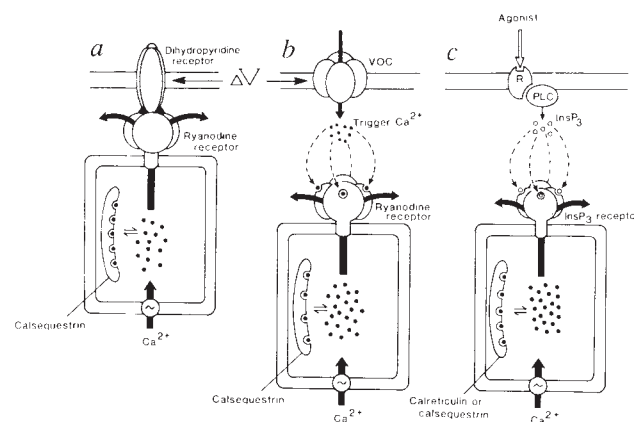


FIG. 2 Control of calcium release by intracellular tetrameric calcium channels. *a*, RyRs located in the sarcoplasmic reticulum of skeletal muscle contribute to the T-tubule foot structure responsible for excitation-contraction coupling. The dihydropyridine receptor in the surface membrane senses a change in voltage ( $\Delta V$ ) and undergoes a conformational change which is transmitted through the bulbous head of the RyR to open the calcium channel in the sarcoplasmic reticulum. *b*, Calcium-induced calcium release in cardiac muscle and perhaps also in neurons. A voltage-operated channel (VOC) responds to  $\Delta V$  by gating a small amount of trigger calcium, which then activates the RyR to release stored calcium. *c*, Agonist-induced calcium release. Signal transduction at the cell surface generates  $\text{InsP}_3$  which diffuses into the cell to release calcium by binding to  $\text{IP}_3\text{Rs}$  (see Box 3).

(>micromolar). Caffeine can also open the RyR channel. These RyRs contribute to calcium signalling in many different cell types (skeletal muscle, cardiac muscle, neurons, chromaffin cells, smooth muscle, pituitary cells and sea urchin eggs).

The remarkable structural similarity that exists between  $\text{IP}_3\text{Rs}$  and RyRs probably reflects a common evolutionary origin which is supported by the very close sequence homology identified primarily in the two membrane-spanning domains and in the free C-terminal region, which includes two cysteines within a TXCFICG motif that is absolutely invariant for all the intracellular calcium channels examined so far (Box 3). This free C-terminal tail may play some part in channel opening because monoclonal antibodies that bind to this region (see Box 3) can either inhibit<sup>35</sup> or enhance  $\text{InsP}_3$ -induced calcium release<sup>36</sup>. The conserved cysteine residues located in this region may also represent the target sites of thiol reagents which enhance the calcium-mobilizing activity of both receptor families (see later).

This structural and molecular homology may account for many of the functional similarities that exist between  $\text{IP}_3\text{Rs}$  and RyRs.

### Intracellular calcium stores

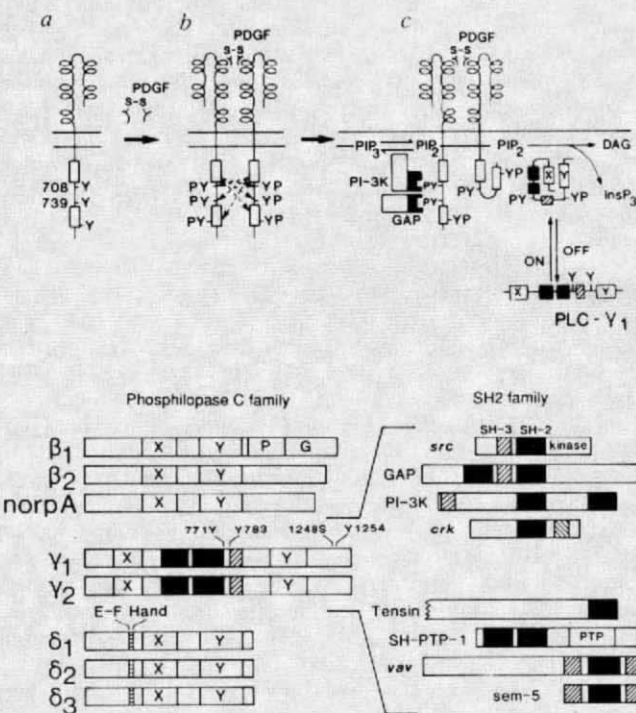
The membrane stores from which calcium is released contain three major components—pumps to sequester calcium, binding proteins (such as calsequestrin and calreticulin) to store calcium and the specific  $\text{IP}_3\text{R}$  or RyR channels to release calcium back into the cytosol (Fig. 2). These receptors are normally located on modified portions of the endoplasmic reticulum (ER)<sup>43</sup>. One such structure, the calciosome, was originally thought to be the  $\text{InsP}_3$ -sensitive store, but recent studies on Purkinje neurons suggest that these organelles may be heterogeneous as some may be sensitive to ryanodine<sup>44</sup>. The distribution of such  $\text{InsP}_3$ - or ryanodine-sensitive calcium stores varies considerably from cell to cell. Some cells either have ryanodine-sensitive stores (skeletal muscle) or  $\text{InsP}_3$ -sensitive stores, as in *Xenopus*

### BOX 2 Signal transduction through tyrosine kinase-linked receptors

TYROSINE kinase receptors (such as PDGF, EGF) generate  $\text{InsP}_3$  and DAG by interacting directly with PLC- $\gamma$ 1, one of the PLC family members (see below). The sequence of events is illustrated using the PDGF receptor as an example. *a*, The receptor consists of a single transmembrane protein containing a cytoplasmic tyrosine kinase which is separated into two (boxes). *b*, PDGF induces dimerization allowing the two kinase domains to phosphorylate each other on specific tyrosine residues (Y) which provide docking sites responsible for interacting with different members of the SH2 family. *c*, An SH2 domain on PLC- $\gamma$ 1 (filled boxes) recognizes and binds to a specific phosphotyrosine residue. Once the PLC- $\gamma$ 1 is phosphorylated on specific residues (see below) it begins to hydrolyse  $\text{PtdIns}(4,5)\text{P}_2$  (labelled here as  $\text{PIP}_2$ ) to give DAG and  $\text{InsP}_3$ .

There are three major PLC family members ( $\beta$ ,  $\gamma$ ,  $\delta$ )<sup>18,19</sup>. An earlier report of a cDNA coding for a PLC- $\alpha$  (ref. 20), which showed no sequence identity to the other PLC enzymes, may not be a PLC but is probably thiol-protein disulphide oxidoreductase<sup>21</sup>. There is no sequence data on a new PLC- $\epsilon$  family<sup>19</sup>. Two sites (P and G) on PLC- $\beta$ 1 are responsible for interacting with  $\text{G}_{\alpha q}$ <sup>11</sup>. There is little sequence homology between PLC- $\beta$ , PLC- $\gamma$  and PLC- $\delta$ , except for domains X (~170 amino acids) and Y (~260 amino acids). PLC- $\beta$  and *norpA* are coupled to G proteins (Box 1), whereas PLC- $\gamma$ 1 interacts with tyrosine kinase-linked receptors (see above) which phosphorylate the enzyme on at least 3 tyrosines (771, 783 and 1254) that are important for activating the enzyme. Phosphorylation of the serine (S) at 1248 by PKC or PKA may be responsible for inhibiting signal transduction<sup>18</sup>. The mode of activation of PLC- $\delta$  is unknown. It may represent a calcium-sensitive form of the enzyme as it contains one canonical EF-hand motif<sup>22</sup>. The activity of PLC- $\delta$ 1 is specifically increased in the aortas of spontaneously hypertensive rats<sup>23</sup>.

The *src* proto-oncogene contains two domains, called *src* homology 2 and 3 (SH2 and SH3), which are found within the structure of many other proteins, particularly those concerned with signal transduction. PLC- $\gamma$  is also a member of this family as it contains two SH2 domains (filled boxes) and one SH3 domain (hatched box). A new family member is a phosphotyrosine phosphatase (SH-PTP-1) containing two SH2 domains. The



SH2 domains function as adaptors that bind to specific tyrosine residues (see above). Located near each phosphorylated tyrosine there is a short sequence motif which determines the specificity of binding to various members of the SH2 family.

oocytes<sup>45</sup>, whereas there are others that contain both (atrial cells, vascular smooth muscle, neurons, chromaffin cells and sea urchin eggs). Cerebellar Purkinje cells have both RY and IP<sub>3</sub>Rs distributed throughout the cytosol on elements of the smooth endoplasmic reticulum (ER) in both the soma and along the dendrites<sup>41,46</sup>. Unlike the RYR, however, the IP<sub>3</sub>R is also found in the spines<sup>41</sup>. In double-labelling experiments, some elements of the endoplasmic reticulum had both receptors. Although there is some physiological evidence to support such a co-localization in some cell types such as PC12 cells<sup>47</sup>, most studies indicate that these two receptors operate separate stores, as in sea urchin eggs<sup>48</sup>. However, these two stores may interact with each other to generate calcium signals.

### InsP<sub>3</sub>-induced calcium release

Calcium contained within intracellular stores is released to the cytosol when InsP<sub>3</sub> binds to its receptor (Fig. 2c)<sup>2,4-6</sup>. This action of InsP<sub>3</sub> has been studied either at the single receptor level, in single cells or in cell populations. When embedded in a lipid membrane, the IP<sub>3</sub>R responds like a conventional channel, displaying an increase in open frequency in response to InsP<sub>3</sub> (refs 49, 50). The mean open time was less than 10 ms and there was evidence of four conductance states, each of ~20 pS (ref. 50). On the basis of finding evidence of co-operativity (Hill coefficients ( $n_H$ )  $\geq 3$ ), Meyer *et al.*<sup>51</sup> proposed that channel opening depends upon InsP<sub>3</sub> binding sequentially to the four putative binding sites of the tetrameric receptor (Fig. 2c). Each binding step could cause a partial opening of the channel in order to account for the different conducting states observed in patch recordings of purified IP<sub>3</sub>Rs (ref. 49). However, others have not found any evidence of co-operativity ( $n_H = 1$ )<sup>50,52</sup> suggesting that binding of a single molecule of InsP<sub>3</sub> is sufficient to account for channel opening. Clearly we still have a long way to go to understand just how InsP<sub>3</sub> acts to open individual channels.

At the next level of organization, the action of InsP<sub>3</sub> has been studied on populations of receptors prepared either in membrane vesicles or in their normal location within permeabilized cells. The response is extremely fast, reaching a maximum within 140 ms of adding InsP<sub>3</sub> to synaptosomes<sup>52</sup>. All these studies have revealed considerable variability in the sensitivity of InsP<sub>3</sub>-induced release, which gives rise to the phenomenon of 'quantal calcium release'<sup>53-56</sup>. As the level of InsP<sub>3</sub> rises, a fixed proportion of the stored calcium is released, with the remainder becoming accessible at higher doses. This variability is evident when studying purified receptors in artificial membranes<sup>56</sup>, calcium release in single cells<sup>55,57</sup> or in cell populations<sup>53-55</sup>. A spatial analysis of calcium release triggered by applying increasing doses of InsP<sub>3</sub> to *Xenopus* oocytes revealed the existence of hot spots, confirming that the InsP<sub>3</sub>-sensitive stores were not uniformly sensitive<sup>57</sup>.

There are two main ways in which the sensitivity of InsP<sub>3</sub>-induced calcium release might vary. First, IP<sub>3</sub>R sensitivity may change depending on the calcium content of the endoplasmic reticulum<sup>38,58-60</sup>. The available evidence suggests that its sensitivity increases as the store charges up with calcium. As there are no obvious calcium-binding domains within those parts of the IP<sub>3</sub>R that face the ER lumen, the calcium-sensing might be mediated by a separate protein with calsequestrin or calreticulin (Fig. 2) being obvious candidates. However, such a mechanism cannot explain the apparent quantal release of calcium by purified IP<sub>3</sub>Rs reconstituted into liposomes<sup>56</sup>. The second proposal, therefore, is that variations in sensitivity may depend upon receptor heterogeneity (Box 2) arising from the presence of different gene products, from alternative splicing or from post-translational modifications such as phosphorylation or autophosphorylation (because the IP<sub>3</sub>R can function as a protein kinase<sup>61</sup>). Phosphorylation of the unspliced neural version results in a decrease in sensitivity<sup>62</sup>, whereas the opposite occurs for the spliced version found in the periphery<sup>63</sup>. As individual cells can express different IP<sub>3</sub>Rs, variations in sensitivity might

arise by varying the contribution of different subunits to the tetrameric receptor complex<sup>29</sup>.

At the heart of the current debate, therefore, is the question of what determines the different sensitivities of IP<sub>3</sub>Rs. Is it a difference in receptor structure or is the variability imposed upon the receptor by the calcium content of the endoplasmic reticulum? It is essential that we resolve this question because it is relevant not only to the process of calcium entry but also to the mechanism of calcium spiking in intact cells (see later).

### Calcium-induced calcium release

The considerable structural and molecular homology shared by RYR and IP<sub>3</sub>R is mirrored in many functional similarities, the most striking of which is a common sensitivity to calcium. Each heartbeat is driven by a regenerative process of calcium-induced calcium release (CICR), when a small influx of calcium through voltage-operated calcium channels triggers an explosive release of stored calcium from the sarcoplasmic reticulum (Fig. 2b). This positive feedback process whereby calcium triggers its own release is a property of RYRs and it may also occur in those non-muscle cells expressing these receptors. As in the heart, calcium entering neurons through voltage-operated channels can be amplified by CICR from internal pools<sup>64</sup> and this can lead to the generation of repetitive calcium spikes<sup>65</sup>. RYRs may also generate calcium spiking in mouse eggs<sup>66</sup> and pancreatic acinar cells<sup>67</sup>. This calcium-sensitive regenerative property of RYRs is also displayed by IP<sub>3</sub>Rs.

Perhaps the most interesting property of InsP<sub>3</sub>-induced calcium mobilization is its all-or-none property manifested as a sudden and near-maximal release of calcium if the level of InsP<sub>3</sub> is gradually increased through injection<sup>68</sup> or by flash photolysis of caged InsP<sub>3</sub> (ref. 57). When visualized by confocal microscopy, the response in *Xenopus* oocytes had two phases. Initially there were small hot spots resulting from the localized release of calcium, which is probably related to the phenomenon of quantal calcium release described earlier. Above a certain threshold, these hot spots suddenly transformed into the all-or-none response that appeared as expanding circular or spiral waves<sup>57,69,70</sup>. This all-or-none response seems to arise through a positive feedback effect whereby calcium stimulates its own release. The IP<sub>3</sub>R displays a bell-shaped response to calcium which thus functions as a coagonist with InsP<sub>3</sub> to release stored calcium<sup>52,71,72</sup>. In the absence of calcium, InsP<sub>3</sub> has little effect, but becomes increasingly active as the concentration of calcium rises, reaching a maximum at about 300 nM, after which it begins to be inhibitory. Using flash photolysis of caged InsP<sub>3</sub> and caged calcium, Iino and Endo<sup>73</sup> demonstrated that these positive and negative effects of calcium operated sequentially during the rising phase of a calcium spike. Positive feedback enhances the initial release, but this is soon curtailed as the accumulation of calcium activates the negative feedback component. The latter may depend upon the receptor switching from a low-affinity (R<sub>L</sub>) state, which can gate calcium, to an inactive high-affinity (R<sub>H</sub>) state<sup>74</sup>. Once the concentration of calcium returns to its resting level, the inactive R<sub>H</sub> state converts back to the sensitive R<sub>L</sub> state in about one second, thus setting the stage for another spike. This calcium-dependent interconversion of the IP<sub>3</sub>R may contribute to the mechanism of repetitive calcium spiking<sup>52,73,74</sup> (see later). Like the RYR, therefore, the IP<sub>3</sub>R also seems to have a CICR mechanism which is of fundamental importance for understanding of how cells generate calcium oscillations and waves.

### Inositol phosphates and calcium influx

Calcium entry into cells can be regulated by a number of mechanisms, for example through channels operated by voltage, by receptors or by second messengers. With regard to the latter, most attention has focused on the inositol phosphates, InsP<sub>3</sub> and InsP<sub>4</sub> (refs. 9, 10). In some cells, there are suggestions that these inositol phosphates may directly activate specific channels



in the plasma membrane. For example, the  $\text{InsP}_3$ -induced entry of calcium in lymphocytes may be mediated by a new  $\text{IP}_3\text{R}$  which contains sialic acid and is localized in the plasma membrane<sup>75</sup>. Similarly, the plasma membrane of olfactory cells seems to have an  $\text{InsP}_3$ -sensitive calcium channel<sup>36,76,77</sup>. When compared with the  $\text{IP}_3\text{Rs}$  on the ER, putative plasma membrane receptors are less specific with regard to  $\text{InsP}_4$  (refs 75, 76). In olfactory cells, for example, the binding protein was equally sensitive to  $\text{InsP}_3$  and  $\text{InsP}_4$  (ref. 76). By contrast, the  $\text{InsP}_4$ -sensitive calcium channel in the plasma membrane of endothelial cells was insensitive to  $\text{InsP}_3$  (ref. 78). There is growing evidence, therefore, that inositol phosphates may have direct effects on calcium channels within the plasma membrane.

In addition to these direct effects,  $\text{InsP}_3$  may stimulate calcium entry indirectly through a more complex mechanism involving the ER. Putney<sup>79</sup> coined the term 'capacitative entry' to introduce the idea that the influx of external calcium seemed to be regulated by the calcium content of a portion of the ER lying close to the plasma membrane. In some cells, calcium entry is stimulated when the ER stores are artificially emptied by applying the calcium pump inhibitors thapsigargin<sup>47,80-82</sup> or 2,5-di-*tert*-butylhydroquinone<sup>82</sup>, or the calcium ionophore ionomycin<sup>81</sup>. When the ER is fully charged, entry is prevented, but as soon as  $\text{InsP}_3$  drains calcium out of these stores, the influx of calcium switches on automatically. Such a mechanism is thought to account for the entry of calcium into lacrimal<sup>80</sup> or mast cells<sup>81</sup> following cell perfusion with  $\text{InsP}_3$ . Others, also using lacrimal cells, failed to see such an  $\text{InsP}_3$ -dependent entry unless they added  $\text{InsP}_4$  (ref. 83). Irvine<sup>10</sup> has presented a detailed analysis of the controversy regarding the role of these two inositol phosphates in regulating calcium entry.

The most difficult aspect to explain concerning this capacitative mechanism is how the calcium content of the ER determines the rate of calcium entry across the plasma membrane. An interesting possibility is that the  $\text{IP}_3\text{R}$  might function to communicate information between the ER and the plasma membrane<sup>9,84</sup>. By analogy with excitation-contraction coupling, in which the large cytoplasmic head of the RYR links the T-tubule to the internal calcium store (Fig. 2a), the large head of the  $\text{IP}_3\text{R}$  may also convey information between the ER and the plasma membrane<sup>9,84</sup>. The idea is that this cytoplasmic head communicates with two calcium channels, one in the ER and the other in the plasma membrane.  $\text{InsP}_3$  binding will induce a conformational state that opens the ER channel, and as the pool loses its calcium, the receptor may change its conformation, leading to an opening of the plasma membrane channel. Irvine<sup>9,10</sup> has argued that the opening of this entry channel may also require  $\text{InsP}_4$  and that such an action would explain those instances where both inositol phosphates are required. As yet there is no information on whether stores regulated by RYRs can induce capacitative entry if their calcium is drained by agents such as caffeine.

In summary, the conformational coupling hypothesis considers that the  $\text{IP}_3\text{R}$  controls the mobilization of both internal and external calcium. Although the mechanism of calcium entry remains a matter for debate, what is not in doubt is that elevated levels of  $\text{InsP}_3$ , perhaps acting together with  $\text{InsP}_4$ , can maintain a constant influx of calcium. In cells that are oscillating, the magnitude of this influx component through second messenger-operated channels is often rather small and has little effect on the intracellular level of calcium during the interval between spikes, mainly because it is rapidly sequestered by the internal stores. But by charging up these stores, the influx mechanism transforms the cytoplasm into an 'excitable medium'<sup>69</sup>, thus setting the stage for the generation of the repetitive calcium spikes and waves described below. In effect, these internal stores integrate this small influx over time before periodically releasing the accumulated signal as a regenerative calcium spike.

### Spatiotemporal aspects of $\text{Ca}^{2+}$ signalling

Now that we are beginning to understand the basic mechanisms of calcium release and entry, the next challenge is to describe the complex spatiotemporal patterns of calcium signalling revealed to us by single-cell imaging techniques. Many of the cells that respond to calcium-mobilizing agonists display a repetitive pattern of calcium spikes whose frequency is sensitive to both agonist concentration and the level of external calcium<sup>2,25,84,85</sup>. In addition to this temporal patterning, each spike often displays a recurring spatial organization. There is a specific initiation locus from which the calcium spreads as a regenerative wave<sup>57,67,69,86</sup>. Secretagogue-induced waves in pancreatic acinar cells initiate in the apical region before propagating towards the basal pole<sup>67</sup>.

Waves propagate through the cytosol using either ryanodine or  $\text{IP}_3\text{Rs}$ . In sea urchin eggs, the fertilization-induced wave spreads around the periphery (Fig. 3)<sup>87</sup>, which corresponds to the cortical location of the RYRs<sup>42</sup>. RYRs are also thought to contribute to wave propagation in pancreatic acinar cells<sup>67</sup> and in mouse eggs<sup>66</sup>, whereas  $\text{IP}_3\text{Rs}$  seem to operate in *Xenopus* oocytes<sup>70</sup>, *Xenopus* eggs<sup>88</sup> and in hamster eggs<sup>35</sup>. In *Xenopus* oocytes that lack RYRs<sup>45</sup>, there are numerous initiation foci (hot spots) and the waves migrate outwards as expanding spheres or spirals which annihilate as they crash into each other<sup>57,69,70,88</sup>. Such spiral waves could be induced by injecting the non-metabolizable analogue  $\text{InsP}_3$  (ref. 70), whereas the injection of calcium had no effect even though it caused a localized elevation of calcium<sup>88</sup>. In the presence of  $\text{InsP}_3$ , however, the injection of calcium was able to initiate waves. Heparin, which is a specific inhibitor of the  $\text{IP}_3\text{R}$ , was found to stop the waves in both *Xenopus*<sup>88</sup> and mouse oocytes<sup>89</sup>. The calcium waves in hamster eggs induced by fertilization or a local injection of  $\text{InsP}_3$  were inhibited by prior injection with the antibody that binds to the C-terminal region of the  $\text{IP}_3\text{R}$  (Box 3)<sup>35</sup>. Although all this evidence suggests a central role for the

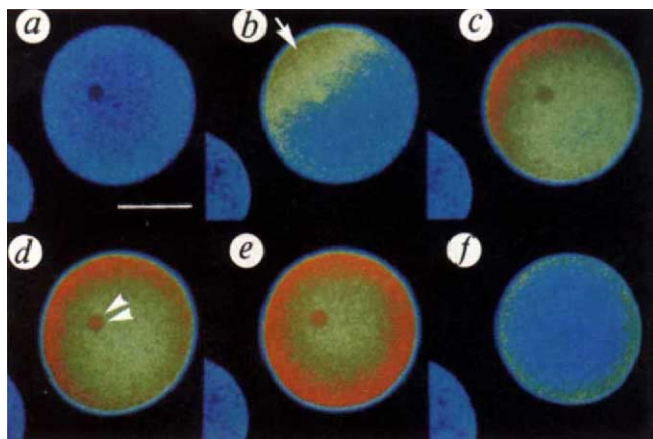


FIG. 3 Fertilization calcium wave in a sea urchin egg observed by confocal microscopy using the fluorescent-indicator fluo-3. The arrow (b) illustrates the initiation point of the calcium wave, which then sweeps around the periphery of the cell (frames c-f). The wave propagated at a rate of  $3\text{--}10\ \mu\text{m s}^{-1}$ . Note the large increase in nuclear calcium (arrowheads in d). (Reproduced with permission from Stricker *et al.*<sup>87</sup>.)

IP<sub>3</sub>R, at least in certain cells, there is controversy concerning the way in which it contributes to the initiation and propagation of calcium waves.

Many ingenious models have been proposed to explain these spatiotemporal aspects of calcium signalling<sup>2,25,84,85</sup>. The emerging consensus is that the basic mechanism requires an element of positive feedback whereby calcium amplifies its own release. One view is that calcium exerts a positive feedback effect on PLC, thereby generating periodic surges of InsP<sub>3</sub> during each spike<sup>85</sup>. In this cross-coupling model, the wave propagates by InsP<sub>3</sub> diffusing from one store to the next, with calcium-induced InsP<sub>3</sub> formation acting to maintain a steep diffusion gradient for InsP<sub>3</sub>. The alternative view is that calcium is the diffusible messenger that enhances its own release through the process of CICR (Fig. 4).

This generalized CICR model has two main components (Fig. 4). An entry mechanism at the plasma membrane, which regulates the supply of calcium to the second component, the oscillator itself, which consists of the calcium stores that have either the RYRs or IP<sub>3</sub>Rs responsible for CICR. The nature of the entry mechanism can also vary: in neural cells it is through voltage-operated channels, whereas in other cells it is usually mediated by the InsP<sub>3</sub>-dependent entry mechanisms described earlier. In the latter case, where entry depends upon a capacitative mechanism based on an InsP<sub>3</sub>-sensitive pool, the model shown in Fig. 4 transforms into the two-pool model described in detail elsewhere<sup>84,90</sup>. The crux of this model, which can be divided into four distinct phases (Fig. 4a–d), is that repetitive spiking depends upon the cyclic release of calcium from internal stores through the regenerative process of CICR. A critical factor appears to be the enhanced influx of external calcium which is taken up by the stores with two consequences. First, the build up of calcium may serve to sensitize the receptor as discussed earlier. A beautiful demonstration of the importance of calcium loading in priming RYRs was described in sympathetic ganglion neurons, where the rapid removal of external calcium could prevent the caffeine-induced spikes right up to the onset of the all-or-none phase (Fig. 4c)<sup>65</sup>. Second, as the stores fill up, their buffering capacity will be reduced, thus leading to a pacemaker elevation of cytosolic calcium (Fig. 4b), which often appears at

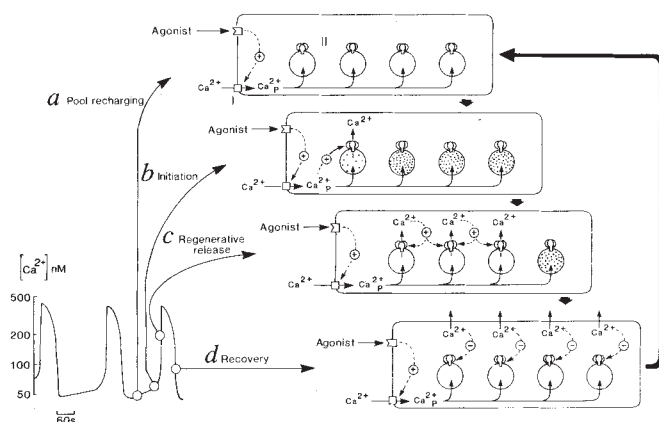


FIG. 4 Calcium-induced calcium release (CICR) model of calcium spiking. The trace on the left represents a typical baseline spiking pattern very similar to that described in cells such as mammalian eggs<sup>66,68</sup> or sympathetic neurons<sup>65</sup>. The model has two main components, calcium entry (I) and the internal stores (II) carrying IP<sub>3</sub>Rs or RYRs, depending on the cell type. a, One of the functions of the external stimulus is to promote an entry of external calcium, often mediated by InsP<sub>3</sub>, to give the primer calcium (Ca<sub>0</sub><sup>2+</sup>) which charges up the internal stores. b, Once these stores are loaded, a process of CICR begins to activate either the IP<sub>3</sub>Rs or RYRs to release calcium. c, Calcium functions as a messenger to release calcium from neighbouring stores to propagate a wave. d, When release ceases, probably owing to a negative feedback effect of calcium, recovery occurs as calcium is pumped out of the cytoplasm (by pumps on the plasma membrane and internal stores) and the cell is ready to begin another cycle.

a specific initiation site where it provides the trigger to detonate the process of CICR (Fig. 4, step c). These two components were clearly demonstrated in *Xenopus* oocytes, in which the response to gradually increasing doses of InsP<sub>3</sub> began with localized elevations of calcium (hot spots) which then transformed into regenerative waves<sup>57</sup>. Dissociation of initiation from propagation probably accounts for abortive spikes such as those observed in HeLa cells responding to low doses of histamine<sup>55</sup>. These initiation sites may represent areas containing calcium stores that are particularly sensitive to activators such as InsP<sub>3</sub>. Finally, the spike terminates through a combination of events. The build up of cytosolic calcium inhibits further release through its inhibitory effect on the release channel and the cytosolic calcium is removed by being pumped back into the stores or out of the cell (Fig. 4d). By measuring the efflux of calcium into a microdroplet surrounding single pancreatic cells, Tepikin *et al.*<sup>91</sup> have found that 15–70% of the calcium spike is extruded from the cell during the recovery phase.

A critical part of this model concerns the change in receptor sensitivity which sets the stage for the initiation of the spike by CICR. Variations in receptor sensitivity could also account for variations in both spike amplitude and rates of wave propagation (M.D. Bootman, personal communication). For example, wave propagation rates in pancreatic acinar cells increased from 57 to 95  $\mu\text{m s}^{-1}$  when the concentration of acetylcholine was raised from 0.1 to 1.0  $\mu\text{M}$ <sup>67</sup>. Lower stimulus intensities will engender fewer sensitized stores and the greater diffusional distances will slow down the wave speeds. Both RYR and IP<sub>3</sub>R display variations in sensitivity. For example, caffeine-induced spiking in neurons results from a lowering of the threshold for CICR. The CICR mechanism evident in eggs at fertilization may be sensitized by a sperm factor<sup>66</sup>. A possible candidate is the cyclic ADP ribose (cADPR)<sup>48</sup> which is formed from NAD<sup>+</sup> (ref. 92). When added to sea urchin egg homogenates, cADPR released calcium from the same stores operated by RYRs. A subthreshold dose of cADPR also sensitized homogenates to the action of ryanodine and caffeine<sup>48</sup>. Just how cADPR acts to sensitize the RYR remains to be shown. A similar change in sensitivity has been described for the InsP<sub>3</sub>-sensitive calcium channels in permeabilized liver cells, which spontaneously released their calcium when the stores were overloaded<sup>58</sup>. During the loading period, the responsiveness of the IP<sub>3</sub>Rs gradually increased until they were able to respond to the ambient level of InsP<sub>3</sub>. IP<sub>3</sub>R sensitivity can also be enhanced by specific thiol reagents, such as oxidized glutathione or thimerosal<sup>58,93,94</sup>, which probably explains how these agents induce calcium spiking in intact hepatocytes<sup>95</sup>, mammalian eggs<sup>96,97</sup> and HeLa cells<sup>94</sup>. The thimerosal-induced calcium spiking in hamster eggs was blocked by the antibody 18A10 (Box 3), confirming the notion that these thiol reagents are acting through the IP<sub>3</sub>R (ref. 97). InsP<sub>3</sub>-sensitive calcium stores can thus operate in two separate modes. They can either respond to a rise in the level of InsP<sub>3</sub>, or they can release their calcium in the presence of a constant level of InsP<sub>3</sub> through a process of CICR.

In summary, when cells are activated, the cytoplasm becomes an excitable matrix which allows a calcium signal to initiate periodically at a specific point before spreading throughout the cell as a regenerative calcium wave. This spatiotemporal organization of calcium signalling seems to depend upon two key processes—influx of calcium across the plasma membrane and a regenerative release of calcium from internal stores controlled by either RYR or IP<sub>3</sub>R.

### Intercellular calcium waves

Waves are not confined to single cells but can travel from cell to cell through two separate mechanisms. In cells lacking gap junctions (clusters of leukaemia cells, for example), waves spread by means of a secreted intermediate (ATP)<sup>98</sup>. The alternative mode of transmission is through gap junctions as described in ciliated epithelial cells of the lung<sup>99</sup> or in glial cell populations



both *in vitro*<sup>100</sup> and *in situ*<sup>101</sup>. Just how the wave crosses the gap junction is unknown, but it could depend upon the diffusion of either calcium itself or  $\text{InsP}_3$ . As the wave reaches the cell periphery, enough calcium may diffuse across to activate the neighbouring cell. Alternatively, the cross-coupling model<sup>85</sup> described earlier would predict a localized calcium-activated elevation of  $\text{InsP}_3$ , which could then diffuse across the gap junction<sup>99</sup>. In the case of *Xenopus* oocytes, there is indirect evidence that  $\text{InsP}_3$  generated in the surrounding follicle cells in response to angiotensin II diffuses across gap junctions to stimulate calcium release in the oocyte<sup>102</sup>. The calcium waves spreading through glial cells may constitute a long-range signalling network acting in concert with conventional neuronal networks. On a more speculative note, an interaction between glial and neuronal oscillators may contribute to the circadian time-keeping mechanism located within the suprachiasmatic nucleus<sup>103</sup>.

### Fertilization and development

The phosphoinositide signalling system is fully established in the gametes and is called upon to regulate major events throughout the life history of a typical cell.  $\text{InsP}_3$  does not appear to play any part in the maturation of invertebrate and amphibian eggs, but it may contribute to the spontaneous maturation of bovine oocytes<sup>104</sup>. The ability of cyclic AMP to inhibit maturation can be overcome by injecting  $\text{InsP}_3$ . During spontaneous maturation, mouse oocytes display  $\text{InsP}_3$ -dependent repetitive calcium spikes for at least two hours, often extending beyond the point of germinal vesicle breakdown<sup>89</sup>. Although the available evidence argues against a role in germinal vesicle breakdown, these calcium spikes might control associated events such as cytoplasmic maturation<sup>89</sup>. Once eggs have matured they remain in suspended animation, awaiting fertilization when the sperm triggers the wave-like surge of calcium (Fig. 3) responsible for stimulating maturation-promoting factor to activate the developmental programme<sup>105</sup>. Just how the sperm triggers the explosive release of calcium in the egg is still something of a

mystery. At the time of fertilization there is an increase in  $\text{InsP}_3$ , and injection of this second messenger can induce some of the early events of fertilization, including the repetitive spiking patterns recorded in mammalian eggs<sup>96,106</sup>. The arguments supporting a role for the  $\text{IP}_3\text{R}$  in propagating calcium waves through *Xenopus* oocytes<sup>70,88</sup> and hamster eggs<sup>97</sup> were summarized earlier. Arguments against a role for a G protein-linked mechanism for generating  $\text{InsP}_3$  have come from experiments in which phorbol esters failed to block fertilization but could inhibit the events activated by injecting GTP- $\gamma\text{S}$  into hamster eggs<sup>106</sup>. An alternative possibility is that the sperm may activate PLC- $\gamma_1$  through a tyrosine kinase-linked mechanism similar to that used by growth factors (Fig. 1). Studies on sea urchin eggs seem to argue against a role for  $\text{InsP}_3$  in fertilization. When injected with heparin there was a normal fertilization-induced calcium transient, whereas the response to either GTP- $\gamma\text{S}$  or  $\text{InsP}_3$  was inhibited<sup>107</sup>. Alternative candidates for a calcium-mobilizing messenger at fertilization include cyclic ADP ribose<sup>48</sup> or cyclic GMP<sup>108</sup>.

Once development begins, the embryo divides rapidly and each mitosis seems to be associated with an increase in calcium. In *Xenopus* embryos, the intracellular level of calcium oscillates during the cell cycle, reaching a peak at mitosis<sup>109</sup>, when it seems to be responsible for triggering nuclear envelope breakdown at prophase<sup>110</sup>. Injection of  $\text{InsP}_3$  can trigger breakdown of the nuclear envelope in sea urchin embryos<sup>110</sup>. A role for this messenger in controlling mitosis has been supported by experiments on *Xenopus* embryos in which injection of heparin or a monoclonal antibody against  $\text{PtdIns}(4,5)\text{P}_2$  was found to arrest the cell cycle<sup>111</sup>. What remains to be determined, however, is how this messenger pathway interacts with maturation-promoting factor to orchestrate an orderly entry into mitosis.

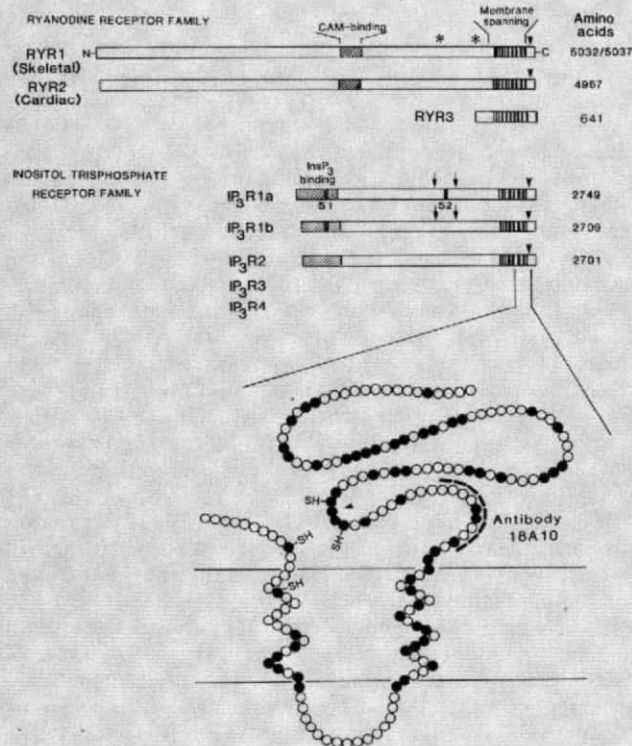
As the embryo grows, the form of the final organism begins to emerge as each cell expresses its specific developmental programme including the establishment of polarity. Both  $\text{InsP}_3$  and DAG have been implicated in setting up the dorso-ventral

### BOX 3 Intracellular calcium channels

The ryanodine receptor (RyR) is almost twice as large as the inositol triphosphate receptor ( $\text{IP}_3\text{R}$ ). The skeletal RyR1 (refs 31, 32) and cardiac RyR2 (ref. 33) display a 66% identity<sup>33</sup>. A much shorter RyR3 has been identified in some non-muscle cells<sup>34</sup>. The  $\text{IP}_3\text{R}_1$  (refs 26, 27) is 70% homologous to  $\text{IP}_3\text{R}_2$  (refs 28, 29). There are partial sequences for the C-terminal domains of two other receptors  $\text{IP}_3\text{R}_3$  and  $\text{IP}_3\text{R}_4$  (refs 28, 29). The primary sequences of members of the two families have regions of homology, particularly in their C-terminal domains which have the membrane-spanning regions. The inset shows the last two membrane-spanning regions and the C-terminal cytoplasmic tail of the skeletal RyR. Filled circles represent amino-acid identities with the  $\text{IP}_3\text{R}_{1a}$ . Dashed line represents the binding site of the monoclonal antibody 18A10, which can inhibit the calcium-mobilizing action of  $\text{InsP}_3$  (ref. 35). Conversely, another antibody directed against this C-terminal region enhances the effects of  $\text{InsP}_3$  (ref. 36). S1 and S2 splice sites: asterisks, cAMP- and calmodulin-dependent phosphorylation sites; arrows, cAMP-dependent phosphorylation sites; arrowhead, highly conserved TXCFICG motif in the free C-terminal domain.

#### Modulation of $\text{IP}_3\text{R}$ activity

Various pharmacological agents can modulate the  $\text{IP}_3\text{R}$  but their actions are not particularly specific. For example, heparin can act as a competitive inhibitor but its usefulness for studies on intact cells is lessened because it may also inhibit the generation of  $\text{InsP}_3$ . The use of caffeine to activate RyRs is complicated by the observation that it is a potent inhibitor of the  $\text{IP}_3\text{R}$  (refs 37, 38). Caffeine abolishes the apparent cooperativity of  $\text{InsP}_3$ -induced calcium release, suggesting that it interferes with the event that couples the binding of  $\text{InsP}_3$  to subsequent channel opening<sup>39</sup>. Finally, ethanol is a potent inhibitor of the  $\text{IP}_3\text{R}$ , which might account for the ataxia associated with ethanol intoxication<sup>40</sup>.



axis in *Xenopus* embryos<sup>112,113</sup>. The level of InsP<sub>3</sub> suddenly increases 2- to 4-fold at the 32-64 cell stage which marks the onset of mesoderm induction when the embryo is most sensitive to the teratogenic effects of Li<sup>+</sup> (ref. 113). An inositol depletion hypothesis attempts to explain this effect of Li<sup>+</sup> on the basis that it inhibits inositol phosphate metabolism, so preventing the regeneration of free inositol<sup>114</sup>. The basic premise is that this depletion of inositol will desensitize phosphoinositide signalling by slowing down the resynthesis of the PtdIns(4,5)P<sub>2</sub> precursor used to release InsP<sub>3</sub>. In keeping with this hypothesis, the spontaneous increase in InsP<sub>3</sub> at the time of mesoderm induction was completely suppressed by Li<sup>+</sup> treatment<sup>113</sup>. Furthermore, Li<sup>+</sup> treatment caused the expected fall in the level of inositol<sup>113</sup>, and the injection of the latter was able to rescue embryos against the teratogenic effect of Li<sup>+</sup> (ref. 112). All this evidence strongly implicates a role for the phosphoinositides in early embryonic induction. Finally, it is of interest to note that expression of the *wnt-1* proto-oncogene can reproduce all the effects of Li<sup>+</sup>, including the duplication of the dorsal axis<sup>115</sup>, the increase in gap junctional communication between ventral cells<sup>116</sup>, and the restoration of an axis when *wnt-1* was expressed on one side of an ultraviolet-irradiated embryo<sup>115</sup>. All these similarities suggest that, like Li<sup>+</sup>, *wnt-1* might modulate the way cells respond to inducing agents by suppressing the level of phosphoinositide signalling.

In the case of mammals, there is indirect evidence to suggest a role for the phosphoinositides in early development. A genetic predisposition for neural tube defects in the curly tail (*ct/ct*) mouse can be overcome by culturing embryos in media containing inositol<sup>117</sup>. Also, there is a hint that the development of normal body asymmetry, namely left-right sidedness, may depend upon this signalling pathway. The *iv* (*situs inversus viscerum*) mutation in mice interferes with this left-right decision-making process so that half of the mutants display *situs inversus* (the normal asymmetry is reversed). This mutant phenotype is reproduced if rat embryos are cultured with the  $\alpha_1$ -adrenergic agonist noradrenaline<sup>118</sup>. As the  $\alpha_1$ -adrenergic receptor is invariably coupled to PLC (Box 1), InsP<sub>3</sub> or DAG are likely candidates to determine left-right handedness during embryonic development.

## Cell growth

Within a fully developed multicellular organism, most cells are non-dividing (G0 phase of the cell cycle), but they retain the option of returning to the cell cycle usually when activated by growth factors. We are entering an exciting phase in which links are being forged between transduction events at the plasma membrane and the cell cycle proteins such as the cyclins and cyclin-dependent kinases which contribute to the DNA-synthesis and maturation-promoting factors which operate at G1/S and G2/M respectively. The role of calcium in stimulating maturation promoting factor at fertilization was described earlier and here I shall concentrate on the G1 events that culminate in activation of DNA synthesis-promoting factor. Other components of this factor are the tumour suppressors (like p53 and the retinoblastoma gene product) and transcription factors such as E2F and DRTF1. During the activation of the DNA synthesis-promoting factor, the inhibitory effect of the suppressors is removed once they become phosphorylated and leave the nucleus (Fig. 5).

The problem of cell cycle control can thus be re-phrased in more precise terms: how do growth factors acting at the cell surface bring about the onset of DNA synthesis by stimulating its promoting factor in the nucleus?

Cell cycle control is complicated by the profusion of growth factors acting on different messenger pathways (Figs 1 and 5). Both G protein-linked receptors (for example, bombesin, bradykinin, endothelin) and tyrosine kinase-linked receptors (for example, PDGF, EGF and insulin-like growth factor (IGF)) can stimulate proliferation. The phosphoinositide-derived sig-

nals InsP<sub>3</sub> and DAG are common to both pathways (Fig. 1), but there is conflicting evidence concerning their role in promoting cell proliferation. In certain cells an increase in phosphoinositide turnover is not in itself a sufficient stimulus to induce mitogenesis<sup>119</sup>. Chinese hamster lung fibroblasts transfected with M1 muscarinic receptors responded to carbachol with an increase in inositol phosphate release and *fos* and *myc* induction, but there was no DNA synthesis<sup>119</sup>. By contrast, the same M1 receptor was mitogenic in CHO cells<sup>120</sup>. The proliferation of glial cells during development of the mammalian brain may depend upon the stimulation of PtdIns(4,5)P<sub>2</sub> hydrolysis by such muscarinic receptors<sup>120</sup>. Proliferation of fibroblasts is inhibited by antibodies directed against PtdIns(4,5)P<sub>2</sub> (ref. 121) or PLC<sup>122</sup>, whereas the injection of either PLC- $\beta$  or PLC- $\gamma$  stimulated DNA synthesis<sup>123</sup>. Furthermore, a toxin extracted from *Pasteurella multocida* is a very effective stimulus of the phosphoinositide system and is a potent mitogen<sup>124</sup>.

Another way of trying to determine whether InsP<sub>3</sub> and DAG have a mitogenic function is to mimic their action with appropriate pharmacological agents. Lymphocytes can be activated using a combination of a phorbol ester or a permeant DAG analogue to activate PKC and an ionophore to mimic the calcium-mobilizing action of InsP<sub>3</sub>. These agents must be present for prolonged periods, consistent with the observation that growth factors are required throughout G1. In hepatectomized rats, DNA synthesis begins at 14 h but can be inhibited by an  $\alpha_1$ -adrenergic inhibitor applied as late as 11 h after partial hepatectomy<sup>125</sup>. Such inhibitors also prevent the increase in calmodulin within the nucleus of liver cells following hepatectomy<sup>126</sup>. Others have shown that calmodulin is required during G1 and may have a role in stimulating DNA synthesis-promoting factor<sup>127</sup>. If calcium influx is reduced during mitogenic stimulation either by lowering the level of external calcium<sup>128</sup> or by using a calcium channel inhibitor<sup>129</sup>, there is a marked inhibition of cell growth.

Measurements of calcium in fertilized eggs<sup>68,97</sup> or lymphocytes<sup>130</sup> following mitogenic stimulation revealed the existence of repetitive calcium spikes which persisted for as long as several hours. As described earlier, InsP<sub>3</sub> plays a central role in driving this oscillatory activity and the wave-like spread of each spike may facilitate the transfer of a calcium signal to the nucleus. Indeed, there are indications that the calcium concentration within the nucleus increases rapidly after antigen stimulation of lymphocytes<sup>131</sup> or fertilization of sea urchin eggs (Fig. 3)<sup>87</sup>. In lymphocytes, antigen activates the InsP<sub>3</sub>/calcium signalling pathway (Fig. 1) that contributes to gene transcription early in G1 (Fig. 5). One of the actions of calcium is to stimulate the translocation of transcriptional factors (NF-AT and NF-IL2A for example) from the cytoplasm into the nucleus. Calcium stimulates the calmodulin-dependent protein phosphatase calcineurin, which is one of the targets for the immunosuppressant drugs cyclosporin A (Fig. 5) and FK-506 (ref. 132). Overexpression of calcineurin makes T cells more resistant to cyclosporin A and FK-506 (ref. 133). Furthermore, this enhanced presence of calcineurin can act in synergy with a phorbol ester to stimulate T cells, thus by-passing the normal calcium requirement<sup>133</sup>.

All this evidence suggests that the InsP<sub>3</sub>/calcium signalling pathway is activated by mitogenic stimuli and may contribute to the changes culminating in DNA synthesis. Activation of transcription factors which then translocate into the nucleus indicates that some of the actions of calcium occur in the cytoplasm. But there is also evidence that the level of nuclear calcium increases dramatically following mitogenic stimulation<sup>87,131</sup>, suggesting that calcium may also act within the nucleus. One of the functions of calcium is to activate MAP II kinase<sup>134</sup>, which is part of the phosphorylation cascade that culminates in the activation of DNA synthesis-promoting factor (Fig. 5).



## Autonomous nuclear signalling

The nucleus contains an autonomous phosphoinositide signalling system every bit as complex as that present at the plasma membrane<sup>135,136</sup>. It contains the lipid kinases necessary to phosphorylate PtdIns to both PtdIns(4)P and PtdIns(4,5)P<sub>2</sub>, which are present both in the nuclear membrane and within the nuclear matrix, which also contains PLC- $\beta$ <sup>137</sup>. What is particularly interesting is that this nuclear PLC- $\beta$  is activated when Swiss 3T3 cells are stimulated by IGF-1, whereas bombesin has no effect even though it is a potent activator of phosphoinositide hydrolysis at the plasma membrane<sup>135,137</sup>. As a result of this hydrolysis, InsP<sub>3</sub> and DAG will be formed in the nucleus where they might perform their familiar second messenger functions. Isolated nuclei display an ATP-dependent uptake of calcium, of which ~20% was subsequently released by InsP<sub>3</sub> (ref. 138). The most likely explanation is that calcium stored within the space between the nuclear membranes was released to the cytosol when InsP<sub>3</sub> bound to receptors on the outer nuclear membrane. But such a mechanism would not explain the increase in nuclear calcium that occurs following activation of lymphocytes<sup>131</sup> or sea urchin eggs<sup>87</sup>. At present there is no evidence for IP<sub>3</sub>Rs on the inner nuclear membrane, so the function of any InsP<sub>3</sub> that might be generated in the nucleus remains a problem.

## Cell transformation

Cancer cells become tumorigenic as a result of multiple independent steps which subvert the normal growth control mechanisms described earlier. Some of these steps have been linked with mutations that either activate proto-oncogenes such as *ras* and *myc*, or remove the inhibitory action of tumour-suppressor genes such as *Rb* and *p53* (Fig. 5). Such tumorigenesis has been induced in cultured cells following transfection with some of the 7-membrane-spanning receptors that are coupled to PLC (Fig. 1 and Box 1) such as 5-HT<sub>1c</sub> (ref. 139), M1, M2 and M5<sup>140</sup>, and  $\alpha_{1B}$ -receptors<sup>16</sup>. Similarly, expression of a mutated  $\alpha_q$  subunit, which enhanced basal PLC activity, was found to transform NIH 3T3 cells but not Rat-1 cells<sup>141</sup>. These receptors and G proteins may be considered as new classes of proto-oncogenes that might transform cells by enhancing the phosphoinositide-derived signals InsP<sub>3</sub> and DAG. For example, the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate), which mimics the action of DAG, can co-operate with the oncogene *ras* to transform primary rat embryo fibroblasts<sup>142</sup>. With regard to calcium, cell transformation leads to a much reduced calcium requirement for growth<sup>143</sup>.

## Neuromodulation and synaptic plasticity

The brain is the richest source of the components of the phosphoinositide signalling pathway. For example, cerebellar Purkinje cells have a very high density of IP<sub>3</sub>Rs and have thus featured significantly in the purification<sup>62</sup>, cloning<sup>26</sup> and fine-structural localization<sup>41,46</sup> of this receptor. The brain has also been important for studying the organization of the PKC family<sup>8</sup>

which is colocalized with the IP<sub>3</sub>R system. The IP<sub>3</sub>R is particularly plentiful in the cerebellum, hippocampus, cerebral cortex, corpus striatum and olfactory tubercule<sup>144</sup>. It is unlikely that the InsP<sub>3</sub>/calcium pathway has any role to play in the rapid signal transfer mediated by ionotropic receptors, but there is growing evidence that many metabotropic receptors employ phosphoinositide-derived signals to modulate both neural activity and the neural plasticity responsible for memory<sup>5</sup>. The recent report that presynaptic mGlu receptors employ phosphoinositide-derived signals as a positive feedback mechanism to enhance glutamate release is particularly interesting<sup>145</sup>.

We still do not know why the large cerebellar Purkinje neurons and CA1 hippocampal neurons have such large numbers of IP<sub>3</sub>Rs. The *staggerer* mutant mouse has a genetic lesion that prevents the expression of IP<sub>3</sub>Rs specifically in the Purkinje neurons, which are poorly developed<sup>146</sup>. Perhaps IP<sub>3</sub>Rs are required for the development of the extensive arborization of the dendritic tree. Another possibility is that the IP<sub>3</sub>R functions to integrate information from the multiple inputs received by this dendritic tree. The co-existence of IP<sub>3</sub>Rs and RYRs may somehow help to integrate information coming in from the outside and to relay it throughout the neuron through the process of CICR already described<sup>64</sup>. Indeed, there is a striking increase in nuclear calcium when neurons are depolarized<sup>147</sup>.

Purkinje and hippocampal neurons are noted for their plasticity because they show long-term changes in neural transmission following certain stimulus regimes. Paired activation of the parallel and climbing fibre inputs results in long-term depression of the parallel fibre-Purkinje cell synapse, which is thought to be mediated through the mGlu receptor that is coupled to InsP<sub>3</sub> formation (Box 1)<sup>148</sup>. The synaptic spines on the Purkinje cells, which receive the input from the parallel fibres, contains an ER system rich in IP<sub>3</sub>Rs<sup>46</sup>. Within the hippocampus, long-term potentiation (LTP) of synaptic transmission can be induced by the large calcium signal resulting from a brief tetanic stimulation of NMDA (*N*-methyl-D-aspartate) receptors. The phosphoinositide signalling pathway may have a role to play because this tetanus-induced onset of LTP was enhanced by activation of the mGlu receptor<sup>149</sup>. Indeed, activation of the mGlu receptor alone can bring about LTP in both the hippocampus<sup>150</sup> and in the dorsolateral septal nucleus<sup>151</sup>. As this mGlu receptor is coupled through a pertussis toxin-sensitive G protein (see Box 1), it is significant that LTP is suppressed by pretreatment with pertussis toxin<sup>152</sup>. The hippocampus receives a large cholinergic innervation which may also play a role because LTP can be enhanced by the cholinesterase inhibitor physostigmine but is reduced by the anticholinergic agent scopolamine<sup>152</sup>. The memory loss we all begin to experience with age may result from a similar impairment of the cholinergic system, which may also be altered in Alzheimer's disease.

This apparent role of the cholinergic system in enhancing synaptic activity seems to be mediated by the phosphoinositide

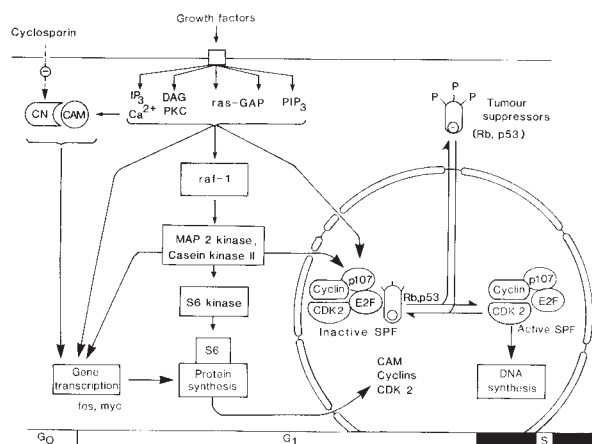


FIG. 5 Summary of signalling pathways operating during G<sub>1</sub>. Growth factors generate a number of putative mitogenic messengers which then feed into various pathways to control both early events at the G<sub>0</sub>/G<sub>1</sub> boundary (for example, *fos* and *myc* transcription) and later events which culminate in the onset of DNA synthesis. One important signalling cascade involves *ras*, *raf* 1 and MAP2 kinase, which can then activate both S6 kinase and DNA synthesis-promoting factor (SPF). SPF contains cyclin, p107, the transcription factor E2F and tumour suppressors such as the retinoblastoma gene product (Rb) and p53. GAP, GTPase-activating protein; CAM, calmodulin; CN, calcineurin; DAG, diacylglycerol; PKC, protein kinase C.

system. The marked potentiation of NMDA responses by acetylcholine in CA1 neurons was reproduced by the flash photolysis of caged  $\text{InsP}_3$  (ref. 153) but was blocked by the  $\text{IP}_3\text{R}$  antagonist heparin<sup>153</sup>. Furthermore, this potentiation was lost in slices prepared from  $\text{Li}^+$ -treated rats but could be restored following addition of free inositol, which is entirely consistent with the inositol depletion hypothesis<sup>114</sup>. Most attention has been focused on the ionotropic receptors (like NMDA and kainic acid receptors), but it is clear that metabotropic receptors operating through the  $\text{InsP}_3$  and DAG messenger systems have a profound effect on neural behaviour, including memory.

## Sensory perception

The phosphoinositide signalling pathway has been adapted for detecting external cues by invertebrate photoreceptors<sup>154</sup>, olfactory sense organs in insects<sup>155</sup>, lobsters<sup>36</sup> and fish<sup>77</sup>, and in certain mammalian taste cells<sup>156</sup>. As for hormonal pathways, these sensory transducing mechanisms use conventional G protein-linked receptors to generate  $\text{InsP}_3$ , for example the rhodopsin-DG $\alpha_q$ -norpA pathway (Box 1) in *Drosophila* photoreceptors. The ER cisternae lying immediately below the photoreceptive microvillar membrane respond to  $\text{InsP}_3$  by releasing calcium, which then opens sodium channels in the surface membrane to give the receptor potential. During prolonged illumination of *Drosophila* photoreceptors, this receptor potential is maintained but decays in transient receptor potential (*trp*) mutants in which the calcium influx component necessary for a maintained response is missing<sup>154</sup>. The *trp* gene encodes a plasma membrane channel with close homology to the dihydropyridine receptor<sup>157</sup>. Minke and Selinger<sup>154</sup> have speculated that the *trp* gene codes for a plasma membrane channel that interacts with an  $\text{IP}_3\text{R}$  to gate calcium through the capacitative mechanism described earlier.

Olfactory and taste organs closely resemble conventional hormonal systems in that they respond to chemical signals by membrane-bound receptors coupled to various second messengers. A role for cyclic AMP in olfactory and taste organs is well-established, but  $\text{InsP}_3$  has also been implicated for detecting certain chemical modalities<sup>158</sup>. Our ability to enjoy sweet substances depends upon the activation of adenylyl cyclase, whereas bitter tastes are detected through  $\text{InsP}_3$  (refs 77, 159). A subpopulation of taste cells in the *circumvallate papillae* have  $\text{IP}_3\text{Rs}$  highly concentrated near the pore where most of the taste receptors are located<sup>156</sup>. Rapid increases in  $\text{InsP}_3$  levels occur in olfactory epithelia in response to pyrazine<sup>159</sup> and in taste cells responding to the intensely bitter substance denatonium<sup>156</sup>. Both electrophysiological<sup>77</sup> and biochemical<sup>76</sup> evidence suggests that  $\text{InsP}_3$  acts on a calcium channel of  $M_r$  107K which is located in the plasma membrane of catfish olfactory cells.

The temporal responsiveness of insect olfactory organs is truly remarkable. Within 50 ms of applying a specific sex pheromone, the  $\text{InsP}_3$  level in cockroach antennae increases from 60 to 2,540 pmol  $\text{mg}^{-1}$  protein<sup>155</sup>. This rapid production of  $\text{InsP}_3$  is fast enough to account for the odorant-induced generator potentials which appear after a latency of 100–200 ms. All this evidence indicates a central role for  $\text{InsP}_3$  in certain forms of sensory perception. It has been suggested that the cyclic AMP and  $\text{InsP}_3$  messenger pathways may interact with each other to enhance odorant discrimination<sup>158</sup>.

## Conclusion

Over the past decade there has been enormous progress in our understanding of how cells use calcium to regulate their activity. The second messenger  $\text{InsP}_3$  has a key role in controlling both the mobilization of internal stores and the entry of external calcium. Cells generate  $\text{InsP}_3$  through two major signalling pathways. In one pathway, receptors are coupled through G proteins to stimulate PLC- $\beta$ , whereas the other pathway depends upon tyrosine kinase-linked receptors which are specifically coupled to PLC- $\gamma$ . When activated, these receptors undergo

autophosphorylation on specific tyrosine residues, providing docking sites that bind to PLC- $\gamma$  and result in its activation.

Once  $\text{InsP}_3$  is released to the cytosol it mobilizes calcium by binding to receptors. Molecular studies have revealed a family of  $\text{IP}_3\text{Rs}$  that have many structural and physiological similarities to RYRs, another major family of intracellular calcium channels. In particular, both families display an autocatalytic process of calcium-induced calcium release which is responsible for both the temporal and spatial patterns of calcium signalling. When cells are stimulated they invariably display regular calcium spikes which often appear as waves. The  $\text{IP}_3\text{R}$  contributes to these complex patterns in two ways. It controls the influx of calcium, which maintains this activity, and it is responsible for the regenerative release of calcium during each spike.

Now that the major aspects of the  $\text{InsP}_3$ -calcium signalling pathway have been mapped out, attention is beginning to focus on its role in specific cellular responses. This pathway is fully operational in germ cells, where it has been implicated in both fertilization and early development. There is continuing support for the hypothesis that  $\text{Li}^+$  re-specifies the dorso-ventral axis in amphibians by repressing the spontaneous surge of  $\text{InsP}_3$  in the early blastula. Once cells differentiate, the  $\text{InsP}_3$ -calcium pathway is adapted to control many responses such as smooth muscle contraction, liver metabolism and stimulus-response coupling in many secretory cells. Many differentiated cells retain the ability to return to the cell cycle when confronted with the appropriate mitogenic stimulus. Many growth factors are capable of stimulating the phosphoinositide signalling pathway but there are conflicting opinions as to its role in regulating cell growth. It seems that the  $\text{InsP}_3$ -calcium and DAG-PKC pathways can contribute to the sequence of events that culminates in DNA synthesis, particularly in primary cells such as lymphocytes, liver and glial cells. In addition, there are also indications that this signalling pathway can induce cells to become tumorigenic.

The nervous system is a rich source of all the components of the phosphoinositide signalling pathway but the function of  $\text{InsP}_3$  and DAG has been difficult to establish. There is evidence that  $\text{InsP}_3$  functions in sensory perception (taste, olfaction and invertebrate photoreception). Perhaps the greatest challenge for the future is to understand how this signalling pathway operates in the CNS. There is already substantial evidence for a role in modulating synaptic plasticity and we can anticipate that both  $\text{InsP}_3$  and DAG will regulate many other neuronal processes. □

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1. Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. *Nature* **306**, 67–69 (1983).
2. Berridge, M. J. & Irvine, R. F. *Nature* **341**, 197–205 (1989).
3. Rana, R. S. & Hokin, L. E. *Physiol. Rev.* **70**, 115–164 (1990).
4. Taylor, C. W. & Richardson, A. *Pharmac. Ther.* **51**, 97–137 (1991).
5. Henzi, V. & MacDermott, A. B. *Neuroscience* **46**, 251–273 (1992).
6. Ferris, C. D. & Snyder, S. H. *J. Neurosci.* **12**, 1567–1574 (1992).
7. Attree, O. et al. *Nature* **358**, 239–242 (1992).
8. Nishizuka, Y. *Nature* **334**, 661–665 (1988).
9. Irvine, R. F. *FEBS Lett.* **263**, 5–9 (1990).
10. Irvine, R. F. *Bioessays* **13**, 419–427 (1991).
11. Wu, D., Jiang, H., Katz, A. & Simon, M. I. *J. Biol. Chem.* (in the press).
12. Blank, J. L., Brattain, K. A. & Exton, J. H. *J. Biol. Chem.* **267**, 23069–23075 (1992).
13. Katz, A., Wu, D. & Simon, M. I. *Nature* **360**, 686–689 (1992).
14. Bernstein, G. et al. *Cell* **70**, 411–418 (1992).
15. Kjelsberg, M. A., Cotechia, S., Ostrowski, J., Caron, M. G. & Lefkowitz, R. J. *J. Biol. Chem.* **267**, 1430–1433 (1992).
16. Allen, L. F., Lefkowitz, R. J., Caron, M. G. & Cotechia, S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 11354–11358 (1991).
17. Simon, M. I., Strathman, M. P. & Gantam, N. *Science* **252**, 802–808 (1991).
18. Rhee, S. G. & Choi, K. D. *J. Biol. Chem.* **267**, 12393–12396 (1992).
19. Cockcroft, S. & Thomas, G. M. H. *Biochem. J.* **281**, 1–14 (1992).
20. Bennett, C. F., Balcarek, J. M., Varrichio, A. & Crooke, S. T. *Nature* **334**, 268–270 (1988).
21. Srivastava, S. P., Chen, N., Liu, Y. & Holtzman, J. L. *J. Biol. Chem.* **266**, 20337–20344 (1991).
22. Baroch, A. & Cox, J. A. *FEBS Lett.* **269**, 454–456 (1990).
23. Kato, H., Fukami, K., Shibasaki, F., Homma, Y. & Takenawa, T. *J. Biol. Chem.* **267**, 6483–6487 (1992).
24. Renard, D. C. et al. *Biochem. J.* **281**, 775–784 (1992).
25. Tsien, R. W. & Tsien, R. Y. *A. Rev. Cell Biol.* **6**, 715–760 (1990).
26. Furuichi, T. et al. *Nature* **342**, 32–38 (1989).



27. Mignery, G. A., Newton, C. L., Archer III, B. T. & Südhof, T. C. *J. biol. Chem.* **265**, 12679–12685 (1990).
28. Südhof, T. C., Newton, C. L., Archer III, B. T., Ushkaryov, Y. A. & Mignery, G. A. *EMBO J.* **10**, 3199–3206 (1991).
29. Ross, C. A., Danoff, S. K., Schell, M. J., Snyder, S. H. & Ullrich, A. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4265–4269 (1992).
30. Mignery, G. A. & Südhof, T. C. *EMBO J.* **9**, 3893–3898 (1990).
31. Takeshima, H. *et al. Nature* **339**, 439–445 (1989).
32. Zorzato, F. *et al. J. biol. Chem.* **265**, 2244–2256 (1990).
33. Otsu, K. *et al. J. biol. Chem.* **265**, 13472–13483 (1990).
34. Giannini, G., Clementi, E., Ceci, R., Marziali, G. & Sorrentino, V. *Science* **257**, 91–94 (1992).
35. Miyazaki, S.-I. *et al. Science* **257**, 251–255 (1992).
36. Fadool, D. A. & Ache, B. W. *Neuron* **9**, 907–918 (1992).
37. Parker, I. & Ivorra, I. *J. Physiol., Lond.* **433**, 229–240 (1991).
38. Missiaen, L., Taylor, C. W. & Berridge, M. J. *J. Physiol., Lond.* **455**, 623–640 (1992).
39. Brown, G. R., Sayers, L. G., Kirk, C. J., Michell, R. H. & Michelangeli, F. *Biochem. J.* **282**, 309–312 (1992).
40. Ilyin, V. & Parker, I. *J. Physiol., Lond.* **448**, 339–354 (1991).
41. Walton, P. D. *et al. J. Cell Biol.* **113**, 1145–1157 (1991).
42. McPherson, S. M., McPherson, P. S., Mathews, L., Campbell, K. P. & Longo, F. J. *J. Cell Biol.* **116**, 1111–1121 (1992).
43. Meldolesi, J., Madeddu, L. & Pozzan, T. *Biochim. biophys. Acta* **1055**, 130–140 (1990).
44. Volpe, P. *et al. EMBO J.* **10**, 3183–3189 (1991).
45. Parys, J. B. *et al. J. biol. Chem.* **267**, 18776–18782 (1992).
46. Satoh, T. *et al. J. Cell Biol.* **111**, 615–624 (1990).
47. Clementi, E. *et al. J. biol. Chem.* **267**, 2164–2172 (1992).
48. Gialione, A., Lee, H. C. & Busa, W. B. *Science* **253**, 1143–1146 (1991).
49. Ehrlich, B. E. & Watras, J. *Nature* **336**, 583–586 (1988).
50. Watras, J., Bezprozvanny, I. & Ehrlich, B. E. *J. Neurosci.* **11**, 3239–3245 (1991).
51. Meyer, T., Wensel, T. & Stryer, L. *Biochemistry* **29**, 32–37 (1990).
52. Finch, E. A., Turner, T. J. & Goldin, S. M. *Science* **252**, 443–446 (1991).
53. Muallem, S., Pandol, S. J. & Beeker, T. G. *J. biol. Chem.* **264**, 205–212 (1989).
54. Taylor, C. W. & Potter, B. V. L. *Biochem. J.* **266**, 189–194 (1990).
55. Bootman, M. D., Berridge, M. J. & Taylor, C. W. *J. Physiol.* **450**, 163–178 (1992).
56. Ferris, C. D., Cameron, A. M., Haganir, R. L. & Snyder, S. H. *Nature* **356**, 350–352 (1992).
57. Parker, I. & Yao, Y. *Proc. R. Soc. Lond. B246*, 269–274 (1991).
58. Missiaen, L., Taylor, C. W. & Berridge, M. J. *Nature* **352**, 241–244 (1991).
59. Tregear, R. T., Dawson, A. P. & Irvine, R. F. *Proc. Roy. Soc. Lond. B243*, 263–268 (1991).
60. Missiaen, L., De Smedt, H., Droogmans, G. & Casteels, R. *Nature* **357**, 599–602 (1992).
61. Ferris, C. D., Cameron, A. M., Brett, D. S., Haganir, R. L. & Snyder, S. H. *J. biol. Chem.* **267**, 7036–7041 (1992).
62. Süpattapone, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 8747–8750 (1988).
63. Burgess, G. M., Bird, G. St. J., Obie, J. F. & Putney, J. W. *J. biol. Chem.* **266**, 4772–4781 (1991).
64. Friel, D. D. & Tsein, R. W. *J. Physiol., Lond.* **450**, 217–246 (1992).
65. Friel, D. D. & Tsein, R. W. *Neuron* **8**, 1109–1125 (1992).
66. Swann, K. *Biochem. J.* **287**, 79–84 (1992).
67. Nathanson, M. H., Padfield, P. J., O'Sullivan, A. J., Burgstahler, A. D. & Jamieson, J. D. *J. biol. Chem.* **267**, 18118–18121 (1992).
68. Miyazaki, S.-I. *J. Cell Biol.* **106**, 345–353 (1988).
69. Lechleiter, J., Girard, S., Peralta, E. & Clapham, D. *Science* **252**, 123–126 (1991).
70. Lechleiter, J. D. & Clapham, D. E. *Cell* **69**, 1–20 (1992).
71. Iino, M. *J. gen. Physiol.* **95**, 1103–1122 (1990).
72. Bezprozvanny, I., Watras, J. & Ehrlich, B. E. *Nature* **351**, 751–754 (1991).
73. Iino, M. & Endo, M. *Nature* **360**, 76–78 (1992).
74. Rouxel, F. P., Hilly, M. & Mauger, R.-P. *J. biol. Chem.* **267**, 20017–20023 (1992).
75. Khan, A. A., Steiner, J. P., Klein, M. G., Schneider, M. F. & Snyder, S. H. *Science* **257**, 815–818 (1992).
76. Kalinoski, D. L. *et al. Biochem. J.* **281**, 449–456 (1992).
77. Restrepo, D., Miyamoto, T., Bryant, B. P. & Teeter, J. H. *Science* **249**, 1166–1168 (1990).
78. Lückhoff, A. & Clapham, E. D. *Nature* **355**, 356–358 (1992).
79. Putney, J. W. *Cell Calcium* **7**, 1–12 (1986).
80. Bird, G. St. J. *et al. Nature* **352**, 162–165 (1991).
81. Hoth, M. & Penner, R. *Nature* **355**, 353–356 (1992).
82. Robinson, I. M., Cheek, T. R. & Burgoyne, R. D. *Biochem. J.* **288**, 457–463 (1992).
83. Chang, L., Gallacher, D. V., Irvine, R. F., Potter, B. V. L. & Petersen, O. H. *J. Membrane Biol.* **109**, 85–93 (1989).
84. Berridge, M. J. *J. biol. Chem.* **265**, 9583–9586 (1990).
85. Meyer, T. & Stryer, L. A. *Rev. Biophys. biophys. Chem.* **20**, 153–174 (1991).
86. Rooney, T. A., Sass, E. & Thomas, A. P. *J. biol. Chem.* **265**, 10792–10796 (1990).
87. Stricker, S. A., Centonze, V. E., Paddock, S. W. & Schatten, G. *Dev. Biol.* **149**, 370–380 (1992).
88. DeLisle, S. & Welsh, M. J. *J. biol. Chem.* **267**, 7963–7966 (1992).
89. Carroll, J. & Swann, K. *J. biol. Chem.* **267**, 11196–11201 (1992).
90. Goldbeter, A., Dupont, G. & Berridge, M. J. *Proc. natn. Acad. Sci. U.S.A.* **87**, 1461–1465 (1990).
91. Tepikin, A. V., Voronina, S. G., Gallacher, D. V. & Petersen, O. H. *J. biol. Chem.* **267**, 14073–14076 (1992).
92. Lee, H. C., Waiseth, T. F., Bratt, G. T., Hayes, R. N. & Clapper, D. L. *J. biol. Chem.* **264**, 1608–1615 (1989).
93. Renard, D. C., Seitz, M. B. & Thomas, A. P. *Biochem. J.* **284**, 507–512 (1992).
94. Bootman, M. D., Taylor, C. W. & Berridge, M. J. *J. biol. Chem.* **267**, 25113–25119 (1992).
95. Rooney, T. A., Renard, D. C., Sass, E. J. & Thomas, A. P. *J. biol. Chem.* **266**, 12272–12282 (1991).
96. Swann, K. *FEBS Lett.* **278**, 175–178 (1991).
97. Miyazaki, S.-I. *et al. FEBS Lett.* **309**, 180–184 (1992).
98. Osipchuk, Y. & Cahalan, M. *Nature* **359**, 241–244 (1992).
99. Boitano, S., Dirksen, E. R. & Sanderson, M. J. *Science* **258**, 292–295 (1992).
100. Charles, A. C., Merrill, J. E., Dirksen, E. R. & Sanderson, M. J. *Neuron* **6**, 983–992 (1991).
101. Dani, J. W., Chernjavsky, A. & Smith, S. J. *Neuron* **8**, 429–440 (1992).
102. Sandberg, K., Ji, H., Iida, T. & Catt, K. J. *J. Cell Biol.* **117**, 157–167 (1992).
103. van den Pol, A. N., Finkbeiner, S. M. & Cornell-Bell, A. H. *J. Neurosci.* **12**, 2648–2664 (1992).
104. Homa, S. T., Webster, S. D. & Russell, R. K. *Dev. Biol.* **146**, 461–472 (1991).
105. Whitaker, M. & Patel, R. *Development* **108**, 525–542 (1990).
106. Swann, K., Igusa, Y. & Miyazaki, S. *EMBO J.* **8**, 3711–3718 (1989).
107. Crossley, I., Whalley, T. & Whitaker, M. *Cell Reg.* **2**, 121–133 (1991).
108. Whalley, T., McDougall, A., Crossley, I., Swann, K. & Whitaker, M. *Molec. Biol. Cell* **3**, 373–383 (1992).
109. Grandin, N. & Charbonneau, M. *J. Cell Biol.* **112**, 711–718 (1991).
110. Twigg, J., Patel, R. & Whitaker, M. *Nature* **332**, 366–369 (1988).
111. Han, J.-K., Fukami, K. & Nuccitelli, R. *J. Cell Biol.* **116**, 147–156 (1992).
112. Busa, W. B. & Gimlich, R. D. *Dev. Biol.* **132**, 315–324 (1989).
113. Maslanski, J. A., Leshko, L. A. & Busa, W. B. *Science* **256**, 243–245 (1992).
114. Berridge, M. J., Downes, C. P. & Hanley, M. R. *Cell* **59**, 411–419 (1989).
115. Sokol, S., Christian, J. L., Moon, R. T. & Melton, D. A. *Cell* **67**, 741–752 (1991).
116. Olson, D. J., Christian, J. L. & Moon, R. T. *Science* **252**, 1173–1176 (1991).
117. Cockcroft, D. L., Brook, F. A. & Copp, A. J. *Teratology* **45**, 223–232 (1992).
118. Fujinaga, M., Maze, M., Hoffman, B. B. & Baden, J. M. *Dev. Biol.* **150**, 419–421 (1992).
119. Pouyssegur, J. & Seuwen, K. A. *Rev. Physiol.* **54**, 195–210 (1992).
120. Ashkenazi, A., Ramachandran, J. & Capon, D. J. *Nature* **340**, 146–150 (1989).
121. Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S. & Takenawa, T. *Science* **239**, 640–643 (1988).
122. Smith, M. R., Liu, Y.-L., Kim, H., Rhee, S.-G. & Kung, H.-F. *Science* **247**, 1074–1077 (1990).
123. Smith, M. R., Ryu, S.-H., Suk, P.-G., Rhee, S.-G. & Kung, H.-F. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3659–3663 (1989).
124. Staddon, J. M., Chanter, N., Lax, A. J., Higgins, T. E. & Rozengurt, E. *J. biol. Chem.* **265**, 11841–11848 (1990).
125. Agell, N., Pujol, M. J., Rius, E. & Bachs, O. *Biochem. biophys. Res. Commun.* **177**, 973–978 (1991).
126. Pujol, M. J., Soriano, M., Aligue, R., Carafoli, E. & Bachs, O. *J. biol. Chem.* **264**, 18863–18865 (1989).
127. Rasmussen, C. D. & Means, A. R. *EMBO J.* **8**, 73–82 (1989).
128. Takuwa, N., Iwamoto, A., Kumada, M., Yamashita, K. & Takuwa, Y. *J. biol. Chem.* **266**, 1403–1409 (1991).
129. Magni, M., Meldolesi, J. & Pandiella, A. *J. biol. Chem.* **266**, 6329–6335 (1991).
130. Wilson, H. A., Greenblatt, D., Poenie, M., Finkelman, F. D. & Tsien, R. Y. *J. exp. Med.* **166**, 601–606 (1987).
131. Yamada, H., Mizuguchi, J. & Nakanishi, M. *FEBS Lett.* **284**, 249–251 (1991).
132. Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I. & Schreiber, S. L. *Cell* **66**, 807–815 (1991).
133. O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J. & O'Neill, E. A. *Nature* **357**, 692–694 (1992).
134. Chao, T.-S.O., Byron, K. L., Lee, K.-M., Villereal, M. & Rosner, M. R. *J. biol. Chem.* **267**, 19876–19883 (1992).
135. Irvine, R. F. & Divecha, N. *Seminars in Cell Biology* **3**, 225–235 (1992).
136. Payrastelli, B. *et al. J. biol. Chem.* **267**, 5078–5084 (1992).
137. Martelli, A. M. *et al. Nature* **358**, 242–245 (1992).
138. Nicotera, P., Orrenius, S., Nilsson, T. & Berggren, P.-O. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6858–6862 (1990).
139. Julius, D., Livelli, T. J., Jessell, T. M. & Axel, R. *Science* **244**, 1057–1062 (1989).
140. Gutkind, J. S., Novotny, E. A., Brann, M. R. & Robbins, K. C. *Proc. natn. Acad. Sci. U.S.A.* **88**, 4703–4707 (1991).
141. De Vito, M., Chen, J., Codina, J. & Iyengar, R. *J. biol. Chem.* **267**, 18263–18266 (1992).
142. Dotto, G. P., Parada, L. F. & Weinberg, R. A. *Nature* **318**, 472–475 (1985).
143. Nicholson, N. B., Chen, S., Blanck, G. & Pollack, R. J. *Cell Biol.* **99**, 2314–2321 (1984).
144. Verma, A., Ross, C. A., Verma, D., Süpattapone, S. & Snyder, S. H. *Cell Reg.* **1**, 781–790 (1990).
145. Herrero, I., Miras-Portugal, M. T. & Sánchez-Rioto, J. *Nature* **360**, 163–166 (1992).
146. Maeda, N., Niinobe, M., Inoue, Y. & Mikoshiba, K. *Dev. Biol.* **133**, 67–76 (1989).
147. Birch, B. D., Eng, D. L. & Kocsis, J. D. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7978–7982 (1992).
148. Blackstone, C. D., Süpattapone, S. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4316–4320 (1988).
149. McGuinness, N., Anwyl, R. & Rowan, M. *Eur. J. Pharmac.* **197**, 231–232 (1991).
150. Bortolotto, Z. A. & Collingridge, G. L. *Eur. J. Pharmac.* **214**, 297–298 (1992).
151. Zheng, F. & Gallagher, J. P. *Neuron* **9**, 163–172 (1992).
152. Ito, I., Okada, D. & Sugiyama, H. *Neurosci. Lett.* **90**, 181–185 (1988).
153. Makram, H. & Segal, M. J. *Physiol.* **447**, 513–533 (1992).
154. Minke, B. & Selinger, Z. in *Progress in Retinal Research* **Vol. 11**, 99–124 (1992).
155. Breer, H., Boekhoff, I. & Tareilus, E. *Nature* **345**, 65–68 (1990).
156. Hwang, P. M., Verma, A., Brett, D. S. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **87**, 7395–7399 (1990).
157. Phillips, A. M., Bull, A. & Kelly, L. E. *Neuron* **8**, 631–642 (1992).
158. Reed, R. R. *Neuron* **8**, 205–209 (1992).
159. Boekhoff, I., Tareilus, E., Strotmann, J. & Breer, H. *EMBO J.* **9**, 2453–2458 (1990).

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# Three-dimensional structure of system I of photosynthesis at 6 Å resolution

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**X-ray structure analysis shows that the monomer of trimeric photosystem I (PS I) of *Synechococcus* sp. consists of a catalytic domain and a smaller domain that connects the monomers. The 4Fe–4S clusters  $F_X$ ,  $F_A$  and  $F_B$ , 28  $\alpha$ -helices and 45 chlorophyll *a* molecules were located. The two large subunits of PS I are represented by nine  $\alpha$ -helices each; they are related by a local 2-fold rotation axis passing through  $F_X$ . Electron densities close to this axis are interpreted as carriers of the electron transfer chain.**

PHOTOSYNTHESIS converts light energy into chemical energy stored in the synthesized organic material. In water-oxidizing photosynthesis, that is in higher plants and cyanobacteria, the primary conversion takes place through light-induced transmembrane charge separations at the reaction centres of photosystems I and II (PS I and PS II). At PS II this takes place by an electron transfer from water at the inner side of the membrane (lumen) to a plastoquinone,  $Q_A$ , at the outer side (stroma). From there the electron is translocated together with a proton across the membrane through a pool of plastoquinones, a cytochrome  $b_6/f$  protein complex and plastocyanin, PC, to the inner side of PS I. At PS I the electron is transferred across the membrane to the outside, where ferredoxin accepts the electron and transports it to  $NADP^+$  reductase.

The vectorial electron transfer and the subsequent proton translocations generate an electrochemical potential across the membrane, which drives ATP synthesis. The products of these primary reactions, NADPH and ATP, are used in the dark reactions to reduce  $CO_2$  to carbohydrates (for review see ref. 1).

Although the reaction centre of photosynthetic purple bacteria with known three-dimensional structure<sup>2,3</sup> is not able to oxidize water, it displays important similarities to that of PS II<sup>3,4</sup>. In contrast, the reaction centre of PS I is related to that in photosynthetic green sulphur bacteria<sup>5,6</sup>, but structural information of PS I at the atomic level is not available. Recently, the genes of the 11 subunits of the thermophilic cyanobacterium *Synechococcus* sp. were sequenced<sup>7,8</sup>. PS I contains two large (A, B of  $M_r \sim 83K$ ) and five small (I, J, K, L, M of 3–15K) membrane intrinsic subunits; in this notation, A corresponds to the gene product *psaA*; B to *psaB* and so on. Further subunits C, D, E, (8–16K) are located on the stromal side and F (15K) on the luminal side of the membrane. The total  $M_r$  of PS I including 90 antenna chlorophylls is 340K.

In PS I, charge separation starts with the photooxidation of the pigment P700 at the luminal side, which may be present as a chlorophyll *a* dimer<sup>9–12</sup>. The released electron moves through  $A_0$  (chlorophyll *a*)<sup>13</sup> and  $A_1$  (vitamin  $K_1$ )<sup>14,15</sup> to the first 4Fe–4S cluster,  $F_X$ , which is bound to the two large subunits A and B. From  $F_X$ , the electron is transferred to two other 4Fe–4S clusters  $F_A$  and  $F_B$ , which are coordinated to subunit C on the stromal side of PS I<sup>16,17</sup>. Here ferredoxin probably takes over the electron for transport to  $NADP^+$  reductase<sup>12</sup>.

The crystallization and X-ray characterization of PS I from different species have been described<sup>18–23</sup>. Crystals of PS I from *Synechococcus* sp. are suitable for X-ray structure analysis to 4 Å resolution<sup>21,24</sup>; here we present the three-dimensional struc-

ture of this PS I at 6 Å resolution. Experimental details are given in Table 1; the overall structure of PS I and its division into two domains are described in Fig. 1*a–c*; sections of the electron density map and their interpretation are shown in Fig. 2*a–c*.

## Structural elements

**4Fe–4S clusters.** In the electron density map (Fig. 2*a*), there are three spherical maxima with density 12 root-mean-square (r.m.s.) deviations above the mean density. They were assigned to the three clusters  $F_X$ ,  $F_A$  and  $F_B$ .

**$\alpha$ -Helices.** The electron density map contains many tubular structures. They were interpreted as  $\alpha$ -helices and fitted with polyalanine  $\alpha$ -helices (Fig. 2*b*) in arbitrary axial orientation, because at 6 Å resolution the polarity of the  $\alpha$ -helices cannot be determined. The  $\alpha$ -helices have not been connected by polypeptide segments because the limited resolution did not permit continuous tracing.

**Chlorophyll *a*.** The electron density map shows numerous disk-like, ellipsoidal globules of 8–10 Å diameter and 4–5 Å thickness. These densities were interpreted as the dihydroporphyrin systems of chlorophyll *a* (Fig. 2*b, c*). The flattened shape of these disks allowed estimation of their orientation in space, but the rotation within their plane is arbitrary.

## Iron–sulphur clusters

The three 4Fe–4S clusters are arranged in the form of an irregular triangle (Figs 2*a*, 3*a*, 4*a*). Its plane roughly passes through the crystallographic 3-fold and local 2-fold axes (Fig. 3*b–d*). The electron density closest to the membrane sheet can be attributed to  $F_X$ , which is supposed to coordinate the membrane-integrated subunits A and B with two cysteines each<sup>12</sup>. The densities of the other two,  $F_A$  and  $F_B$ , are 14 and 21 Å from  $F_X$ , respectively (Fig. 4); distinction between  $F_A$  and  $F_B$  cannot be made. The comparable electron densities of  $F_X$ ,  $F_A$  and  $F_B$  contradict the notion that one of the clusters occurs as 2Fe–2S (ref. 25) and supports recent data that are in favour of three 4Fe–4S (refs 26, 27).

We have compared the distance between  $F_A$  and  $F_B$  (12 Å), which are coordinated to the ferredoxin-like subunit C, with that of the two 4Fe–4S clusters in the known three-dimensional model of bacterial ferredoxin<sup>28,29</sup> from *Peptococcus aerogenes*. Although the polypeptides of the two proteins are different, there is structural analogy because the distances between the two 4Fe–4S clusters are identical. Because this is expected to lead to similar electron transfer rates, provided the polarities of



the redox centre environments and free energies are roughly comparable<sup>30</sup>, the electron transfers of bacterial ferredoxin and subunit C of PS I may be functionally related.

### Arrangement of $\alpha$ -helices

The large number of 28 identified  $\alpha$ -helices is in agreement with circular dichroism (CD) spectroscopic studies, which predicted PS I from algae to contain about 55%  $\alpha$ -helical and only 7%  $\beta$ -sheet structure<sup>31</sup>. Although most of the  $\alpha$ -helices are straight, some are kinked and made up of two pieces (Fig. 3). Most of the helices are of the transmembrane type, as predicted for PS I<sup>12</sup> and oriented at an angle between 3° and 30° to the membrane normal. Seven helices are nearly parallel to the membrane layer, and located towards the stromal or luminal side.

Looking onto the plane of the membrane (Fig. 3d), an elongated array of helices is recognized. Eight transmembrane helices (*a* to *h*) are symmetrically related with eight other helices (*a'* to *h'*) so that *a* corresponds to *a'*, *b* to *b'*, and so on. This defines a local roughly 2-fold rotation axis passing through  $F_X$ . We assign these blocks to subunits A and B because of the high homology of their amino-acid sequences<sup>7</sup>, and because of the folding into a comparable pattern of 11 helices each, as deduced tentatively from hydropathy profiles<sup>8</sup>. The hydropathy profile of subunit A indicates a unique long  $\alpha$ -helix<sup>8</sup>. Because the kinked helix *g* is by far the longest in the array, we tentatively assign  $\alpha$ -helices (*a* to *h*) to subunit A. The division of the array of helices into two blocks (*a* to *h* and *a'* to *h'*) is based on their 'obvious' spatial separation, but other divisions which still obey the approximate symmetry are possible: for example, A (*a* to *d*, *e'*, and *f*, *g*, *h*) and B (*a'* to *d'*, *e*, and *f'*, *g'*, *h'*).

On the luminal side there are two further helices (*n*, *n'*) (Fig.

3d) with an 11 Å shortest separation between their axes, which are also related by the local symmetry and probably belong to subunits A and B. Helices (*i*, *j*, *k*, *l*, *m*) have no symmetry equivalent (Fig. 3d). Helix *i* is connected by electron density to the stromal side of PS I. It cannot belong to the stroma subunits C, D or E, because these can be removed by chaotropic reagents and are probably not anchored by a membrane-intrinsic helix<sup>12</sup>. Helix *i* could perhaps belong to subunit J, which has an amino-terminal transmembrane helix and a hydrophilic region at the carboxy terminus.

Because *j* and *k* are linked by electron density, they are presumably part of subunit K, which possibly consists of two  $\alpha$ -helices<sup>7,8</sup>. Helices *j* and *k* are close to the 3-fold axis in a tight bundle which might be responsible for the organization of the monomers into the trimeric form. For helices *l*, *m* and the additional helices on stromal and luminal sides (white helices only shown in Fig. 3a-c), no definite assignment can be made.

### Arrangement of chlorophyll *a* molecules

Most of the identified 45 chlorophyll *a* molecules are oriented with their dihydroporphyrin planes roughly perpendicular to the membrane (Fig. 3a, b), similar to those observed in the light-harvesting complex II (LHC II)<sup>32</sup> and as predicted by linear dichroism spectroscopic studies<sup>33</sup>. Some of the chlorophyll *a* molecules belong to the electron transfer chain, all others to the antenna system. The latter are located around the transmembrane helical core of the subunits A, B (see Fig. 3a, b). The distances between centres of neighbouring dihydroporphyrins of most of these light-harvesting chlorophyll *a* are 8 to 15 Å, similar to those reported for other light-harvesting complexes<sup>32,34</sup>. Several of the closely spaced chlorophyll *a* form

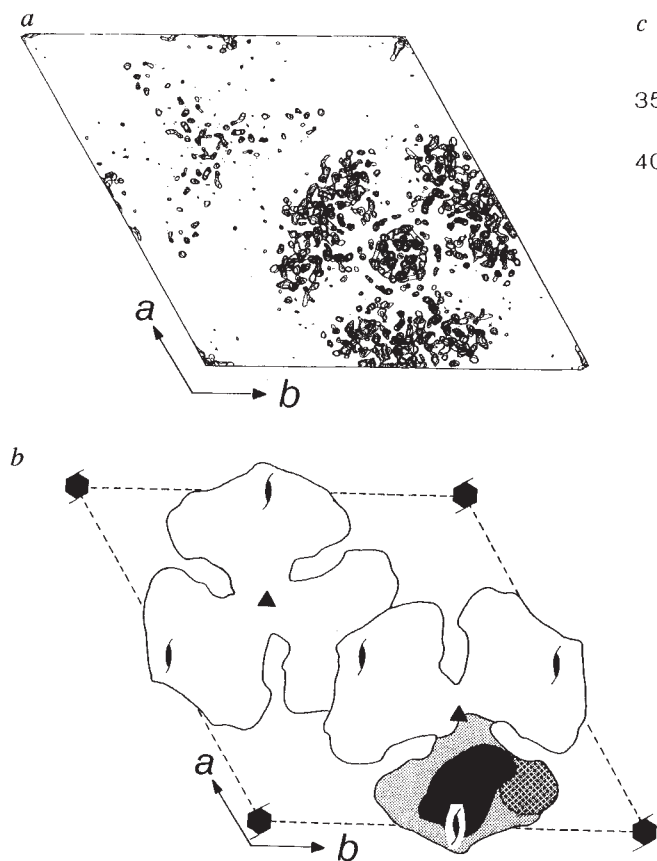


FIG. 1 a, Electron density map. 'Top' view onto the crystallographic *a*, *b*-plane, which is assumed to be parallel to the membrane layer. Shown is a section of 28 Å thickness, which contains a region of the membrane embedded part of the PS I complex. The contours are drawn at a level 2 r.m.s. deviations above the mean density. b, Packing of the PS I trimers in the crystal unit

cell with symmetry elements indicated in the same view as in a. One monomer is shaded, with stromal part black, membrane-embedded part light, and luminal, less well ordered part dotted. The separation line between the monomers is drawn arbitrarily. c, View onto one PS I monomer. Left, view direction down the crystallographic *b*-axis for the monomer which is shaded in b. Right, view direction perpendicular to the crystallographic *b*, *c*-plane. a, b, Arrangement of the PS I trimer in the unit cell. The tripartite disk with ~100 Å radius is centred on the crystallographic 3-fold axis. Each of the three identical monomers is divided into a small and a large domain (c, left) which are connected by narrow sections of electron density (a). The smaller 'connecting' domains with ~40 Å height and ~30 Å width are so close to the 3-fold axis that they appear to be engaged in trimerization. The larger 'catalytic' domain with 130 Å length and 70 Å width harbours the electron transport chain (see below). Because most of the 28 helices that were identified are oriented roughly parallel to the *c*-axis and located within a sheet parallel to the *a*, *b*-plane (Fig. 2a), which is ~40 Å thick (see below) as commonly observed for biological membranes<sup>44</sup>, we associate this sheet with the membrane-embedded part of PS I (c). We can also identify the stromal and luminal sides of the PS I trimer, because the three 4Fe-4S clusters of PS I are known to be located on the stromal side<sup>11</sup> and have been found within the 35 Å-high 'hump' protruding from the large domain (black in b, c). On the luminal side of the large domain, there is a 10 Å depression. On one side of this depression, a large portion of partly connected, less well defined electron density is found (dotted in b, c). A similar outer structure of PS I has previously been observed also by electron microscopy<sup>45-49</sup>, but the partition into two domains was not obvious. Possibly, the observed trimer also occurs *in vivo*<sup>50,51</sup>. In solution, the trimer has been dissociated into dimers and monomers<sup>48</sup>. Because each of the monomer contains one photocentre P700, it represents the PS I.

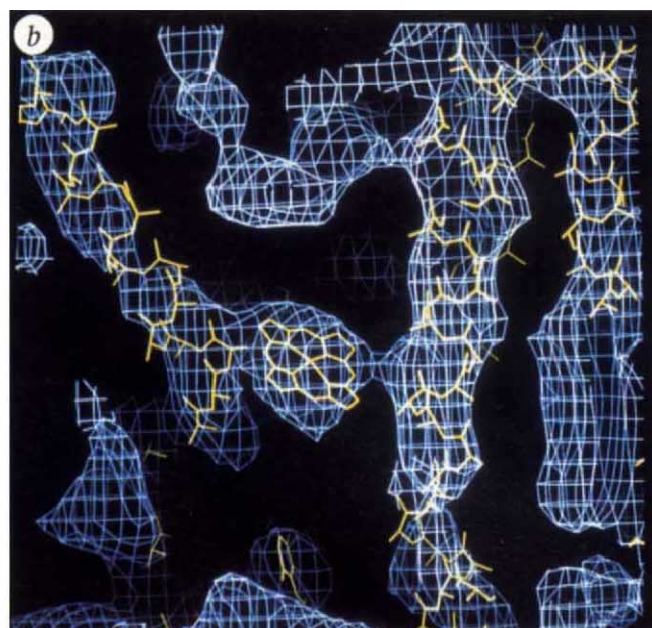
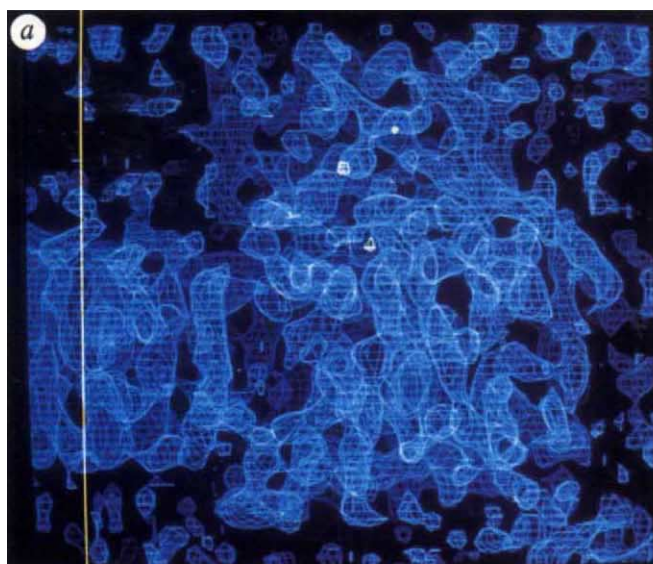


TABLE 1 Data collection and phasing statistics

| Data collection                                           |                    |                 |       |                                |       |                                     |       |                                |        |
|-----------------------------------------------------------|--------------------|-----------------|-------|--------------------------------|-------|-------------------------------------|-------|--------------------------------|--------|
| Data                                                      | Number of crystals | Resolution (Å)  |       | Number of measurements         |       | Unique reflections (% completeness) |       | $R_{\text{merge}}^{\S}$        |        |
| Native*                                                   | 2                  | 20.0–6.0        |       | 49,832                         |       | 18,703 (94.0)                       |       | 0.070                          |        |
| EMTS (1)*†                                                | 1                  | 20.0–6.0        |       | 35,568                         |       | 17,103 (89.8)                       |       | 0.101                          |        |
| EMTS (2)*†                                                | 2                  | 20.0–6.0        |       | 64,035                         |       | 17,714 (91.7)                       |       | 0.058                          |        |
| PIP*‡                                                     | 2                  | 20.0–6.0        |       | 48,973                         |       | 17,657 (91.6)                       |       | 0.130                          |        |
| K <sub>3</sub> UO <sub>2</sub> F <sub>5</sub> ‡           | 1                  | 20.0–9.0        |       | 5,577                          |       | 3,873 (72.4)                        |       | 0.063                          |        |
| Phasing statistics                                        |                    |                 |       |                                |       |                                     |       |                                |        |
| Derivative                                                | Anomalous data     | Number of sites |       | $R_{\text{deriv}}^{\parallel}$ |       | $R_{\text{Cullis}}^{\P}$            |       | Phasing power (resolution (Å)) |        |
| EMTS (1)                                                  | yes                | 3               |       | 0.151                          |       | 0.655                               |       | 1.70 (6.0)                     |        |
| EMTS (2)                                                  | yes                | 4               |       | 0.097                          |       | 0.672                               |       | 1.71 (6.0)                     |        |
| PIP                                                       | yes                | 5               |       | 0.185                          |       | 0.737                               |       | 1.54 (6.0)                     |        |
| K <sub>3</sub> UO <sub>2</sub> F <sub>5</sub>             | no                 | 2               |       | 0.127                          |       | 0.614                               |       | 1.03 (9.0)                     |        |
| Mean figure of merit as a function of resolution (20–6 Å) |                    |                 |       |                                |       |                                     |       |                                |        |
| Resolution range (Å)                                      | 15.48              | 12.63           | 10.67 | 9.23                           | 8.14  | 7.27                                | 6.58  | 6.00                           | total  |
| Number of reflections                                     | 619                | 966             | 1,378 | 1,895                          | 2,472 | 3,109                               | 3,829 | 4,435                          | 18,703 |
| Mean figure of merit                                      | 0.75               | 0.76            | 0.75  | 0.73                           | 0.69  | 0.67                                | 0.61  | 0.54                           | 0.65   |

*Synechococcus* sp. PS I was isolated and purified in trimeric form as described<sup>19</sup>. Crystallization of the trimers was done by the reverse of the 'salting in' procedure. For this, the salt concentration in a solution containing 30 mg ml<sup>-1</sup> protein, 100 mM MgSO<sub>4</sub>, 5 mM 2-(*N*-morpholino)ethanesulphonic acid, pH 6.4, 0.02% w/w β-dodecylmaltoside was slowly reduced within 1–2 days through dialysis against the same buffer with lower salt concentration. At about 6 mM MgSO<sub>4</sub>, crystallization takes place<sup>24</sup>. Crystals grow as hexagonal, dark green prisms (1–2 mm long and 0.5 mm wide). Because the X-ray reflections obtained from these crystals are closely spaced owing to the large hexagonal unit cell with  $a=b=287$  Å and  $c=167$  Å, and diffuse owing to a relatively large mosaic spread ( $\sim 0.5^\circ$ ), interpretable diffraction patterns could only be obtained with the intense and well collimated radiation from synchrotron sources (see below). The space group is  $P6_3$  with two PS I trimers in the unit cell or one PS I monomer in the asymmetric unit. On this basis, the solvent content including detergent is calculated to be 78%. The crystal structure was determined by multiple isomorphous replacement including anomalous dispersion effects. X-ray diffraction data were collected at room temperature (20 °C) \* with an image plate detector at the EMBL-outstation (DESY, Hamburg/Germany),  $\lambda=0.96$  Å, and ‡ with a FAST detector from Enraf-Nonius, Delft/Holland at SRS (Daresbury/United Kingdom),  $\lambda=0.89$  Å. Wavelengths around 1 Å were chosen to prolong the lifetime of the crystals in the X-ray beam. Image plate data were processed using MOSFLM (Imperial College, London) and CCP4<sup>39</sup> programs. FAST data were processed using MADNES<sup>46</sup> and scaled in 3° batches. The  $x, y$  coordinates of all the heavy atoms were derived from difference Patterson maps. Because space group  $P6_3$  is polar, the  $z$ -coordinate of one position of the EMTS(1) derivative was set to  $z=0$ , and the  $z$ -coordinates of the other positions were obtained from the Patterson cross-peaks. These coordinates were used to calculate cross-phase difference Fourier maps of the other heavy-atom derivatives, and the corresponding heavy-atom positions were located. Heavy-atom parameters were refined against centric data, initial phase angles were calculated using PHARE (mean figure of merit 0.65) and served to calculate an electron density map. The phase angles were improved considerably by eight cycles of solvent flattening<sup>41</sup> (mean figure of merit 0.80) using a conservative solvent content of 75% and a 22 Å averaging radius to calculate the protein molecular envelope with the reciprocal-space equivalent<sup>42</sup> of B. C. Wang's algorithm<sup>41</sup>. The electron density map calculated from these improved phases was interpreted with FRODO<sup>43</sup> on an Evans & Sutherland ESV display unit. The atomic coordinates will be deposited with the Brookhaven Protein Data Bank. † EMTS, sodium-2-ethylmercurithiosalicylate; PIP, Bis( $\mu$ -iodo-ethylenediamine-platinum(II))nitrate. Heavy-atom derivatives were prepared by soaking crystals at 1 mM concentrations of the respective compounds for 24 h (EMTS(1) and PIP) and 12 h (EMT(2)); K<sub>3</sub>UO<sub>2</sub>F<sub>5</sub> was at 1 mM and 20 h soak time. §  $R_{\text{merge}} = \sum_h \sum_i |I_i - \langle I \rangle| / \sum_h \sum_i I_i$ ; where  $I_i$  are intensity measurements of a reflection and  $\langle I \rangle$  is the mean intensity of this reflection. ||  $R_{\text{deriv}} = \sum |F_{\text{PH}} - F_P| / \sum |F_P|$ ; where  $F_{\text{PH}}$  and  $F_P$  are structure amplitudes of derivative and native crystal, respectively. ¶  $R_{\text{cullis}} = \sum ||F_{\text{PH}}(\text{obs}) \pm F_P(\text{obs}) - F_H(\text{calc})| / \sum |F_{\text{PH}}(\text{obs}) - F_P(\text{obs})|$ ; for centric reflections. # Phasing power is defined as  $F_H(\text{r.m.s.})/E(\text{r.m.s.})$ ; where  $F_H(\text{r.m.s.})$  are the r.m.s. heavy-atom structure factor amplitudes and  $E(\text{r.m.s.})$  are the r.m.s. residual lack of closure errors.



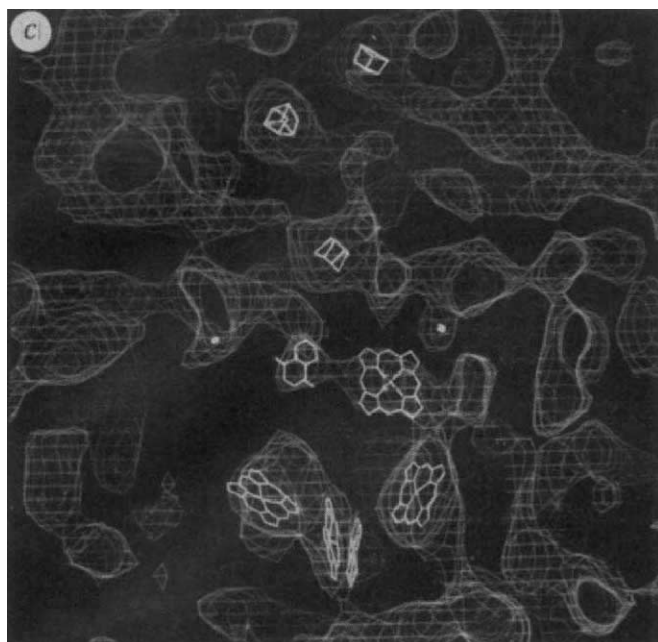
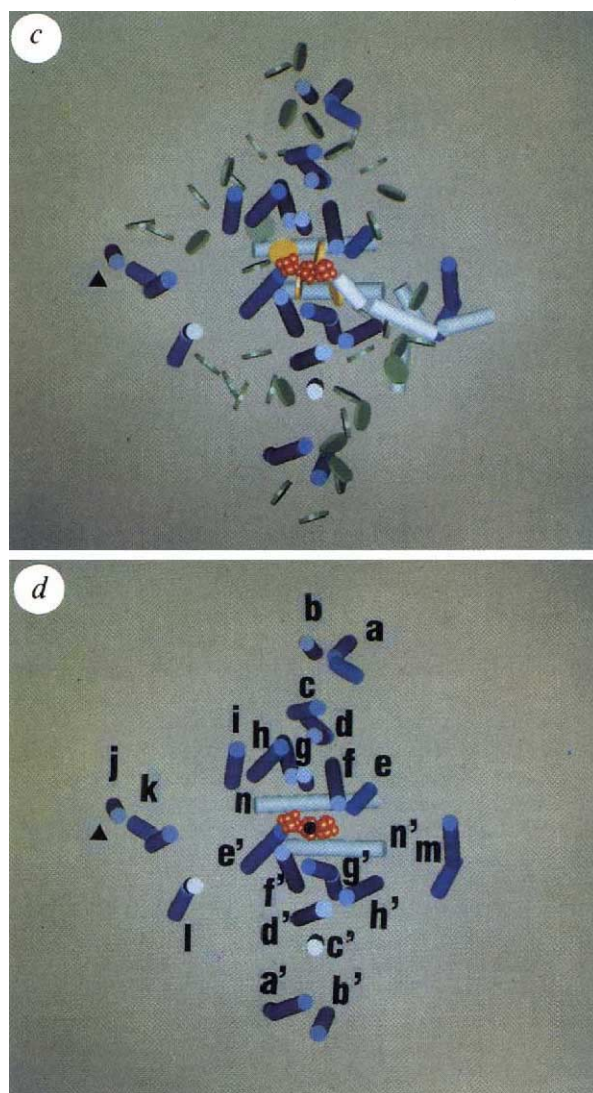
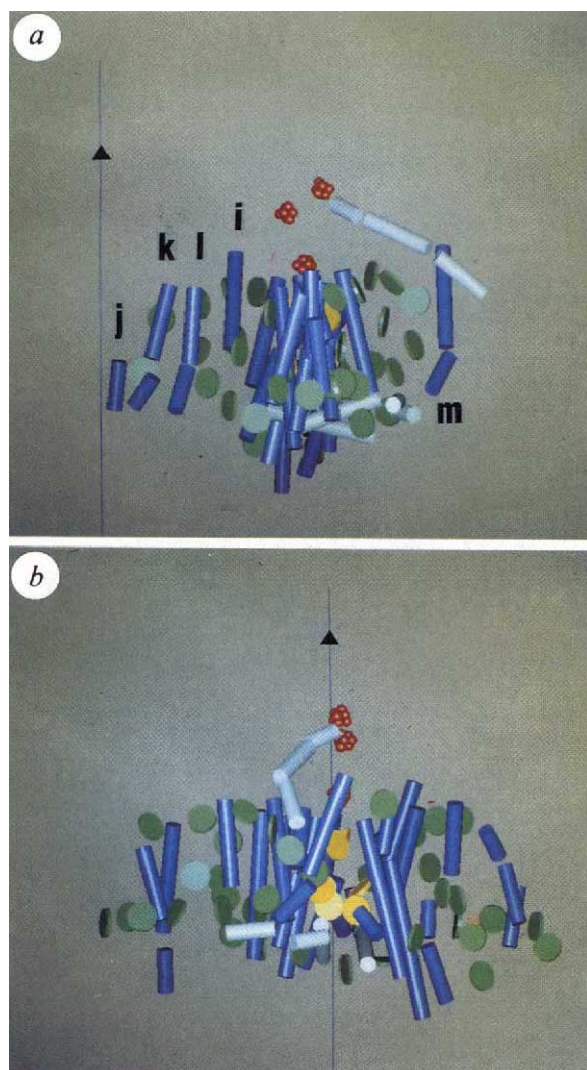


FIG 2 *a*, Section of the electron density map of a PS I monomer with view direction as in Fig. 1*c*, left. Shown is a slab containing the crystallographic 3-fold axis (yellow line). The blue contours are at a level 1.2 r.m.s. deviations above the mean density. The yellow contours (10 r.m.s. deviations) indicate the positions of the three iron-sulphur clusters. *b*, Elements of the electron density map fitted with polyaniline  $\alpha$ -helices and the dihydroporphyrin of chlorophyll *a*. *c*, Section of the electron density map of the electron transfer chain. For the assignments see text. The two white dots indicate possible alternative positions for vitamin  $K_1$ . Contours in *b*, *c* are at the same level as the blue one in *a*.

FIG. 3 Arrangement of structural elements in the PS I monomer pictured with the graphics program O<sup>52</sup>. The crystallographic 3-fold axis is indicated by  $\blacktriangle$ . *a*, View direction as in Fig. 1*c*, left. Transmembrane helices are shown as blue, and horizontal helices as white cylinders. Head groups of the antenna chlorophylls are shown as green disks, electron carriers in yellow.  $F_X$ ,  $F_A$ ,  $F_B$  are indicated as red spheres. Stroma is 'above', lumen 'below' the PS I complex. *b*, As *a*, but rotated 90° about the vertical. *c*, As *a*, but rotated 90° about the horizontal. The stromal side is toward the viewer. *d*, Same as *c*, but for clarity the chlorophylls and helices which are not of the transmembrane type (with the exception of *n* and *n'*) have been omitted. The local roughly 2-fold axis that relates helices *a* to *h* and *a'* to *h'* in subunits A and B is indicated by  $\bullet$ . For the letters see text.



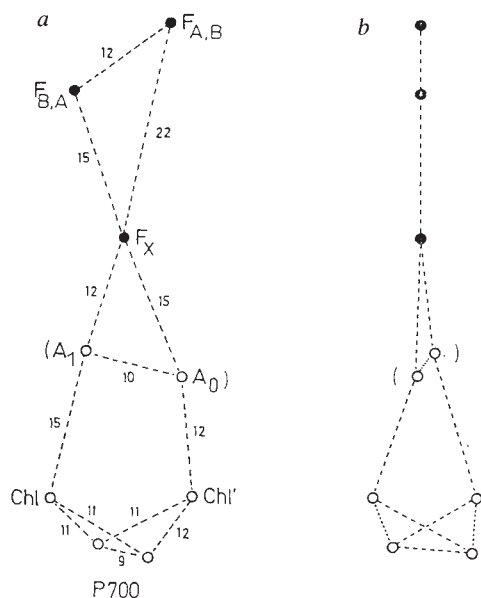


FIG. 4 *a*, Arrangement of the geometrical centres of the electron carriers shown in Fig. 2*c* (distances in Å). View direction is perpendicular to the plane defined by  $F_X$ ,  $F_A$ ,  $F_B$ . The local roughly 2-fold axis is vertical and passes through  $F_X$ . The open circles are meant to indicate the tentative character of the assignments. *b*, Same view as above, but rotated 90° about the vertical.

chains running between the stromal and luminal sides of PS I. Half of the possibly 90 chlorophyll *a* molecules have been located; 12 to 16  $\beta$ -carotene molecules considered to be located in the A and B subunits<sup>12</sup> could not be identified.

### The electron transfer chain

Of particular interest is the electron transfer chain (Figs 2*c* and 4). Because charge separation is directed across the photosynthetic membrane<sup>35</sup>, P700 and the different electron acceptors are expected to form a chain extending from the luminal to the stromal side of PS I. The positions of the terminal acceptors in this chain, the three iron-sulphur centres, could be determined unambiguously; as a consequence, we anticipated that the other electron carriers are located in the region between  $F_X$  and the luminal surface of PS I, and close to the local 2-fold axis passing through  $F_X$ .

The charge separation is initiated at the primary electron donor P700, which might be a chlorophyll *a* dimer<sup>9-12</sup>. In the electron density map (Fig. 2*c*), two chlorophylls located close to the 2-fold rotation axis are tentatively attributed to P700. Their centres are 9 Å apart (Fig. 4). The dihydroporphyrin plane of one of these two molecules is clearly oriented perpendicular to the membrane plane, but the orientation of the second chlorophyll *a* cannot be determined as the corresponding electron density is almost spherical. The orientation shown in Fig. 2*c* would agree with electron paramagnetic resonance (EPR) measurements, which indicate that P700 is oriented perpendicular to the photosynthetic membrane<sup>36</sup>.

The predicted locus of P700 is close to the innermost tip of a distinct 10 Å indentation (Fig. 1*c*) of the luminal surface of the PS I complex and separated from it by the two  $\alpha$ -helices ( $n$ ,  $n'$ ) (Fig. 3*d*). This would be the place to dock plastocyanin. On one side of this docking site, the electron density map is characterized by disordered protein. This appears to be the location of the 15.1K subunit F, which was found by cross-linking studies to interact with plastocyanin<sup>37</sup>.

Two ellipsoidal electron densities in the vicinity of P700 are possibly additional chlorophyll *a* molecules, Chl and Chl', which as yet have not been considered in the electron transfer chain. The centres of these dihydroporphyrins are ~11 Å from the P700 chlorophyll *a* and their planes are strongly inclined towards the normal of the membrane (Fig. 2*c*). This arrangement of the P700 dimer and two Chl and Chl' shows similarities to the RC of the purple bacteria<sup>2</sup>.

Farther 'up' the chain, there is electron density which might be assigned to the electron acceptor  $A_0$  (a chlorophyll *a*). About

10 Å away from  $A_0$ , there is less extensive electron density that corresponds more closely to the naphthoquinone head group of  $A_1$  (vitamin  $K_1$ ). Two features in the electron density map at the locus indicated by white dots (Fig. 2*c*) possibly represent alternative positions for two vitamin  $K_1$  molecules.

The distances between the putative electron carriers (Fig. 4*a*, *b*) predict a unidirectional P700- $A_0$ - $A_1$ - $F_X$ - $F_{A,B}$  electron transfer with  $F_X$  as unambiguous intermediate. This was recently supported by an analysis of the kinetics of the reduction of  $F_X$ , which is independent of the presence and absence of  $F_{A,B}$ <sup>38</sup>.

It must be stressed that our interpretation is tentative and thus at the present level of resolution an unambiguous decision on the arrangement of the electron carriers located between P700 and  $F_X$  is not possible. This refers especially to our assignment of electron densities to  $A_0$  and  $A_1$  and to the notion that there are in fact two vitamin  $K_1$  molecules in PS I<sup>13</sup>. □

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- Witt, H. T. *New J. Chem.* **11**, 91-101 (1987).
- Deisenhofer, J., Epp, O., Miki, K., Huber, R. & Michel, H. *Nature* **318**, 618-624 (1985).
- Deisenhofer, J. & Michel, H. *EMBO J.* **8**, 2149-2170 (1989).
- Trebst, A. *Z. Naturforsch.* **41c**, 240-245 (1986).
- Mathis, P. *Biochim. biophys. Acta* **1018**, 163-167 (1990).
- Nitschke, W., Feiler, J., Lockau, W. & Hauska, G. *FEBS Lett.* **218**, 283-286 (1987).
- Mühlenhoff, U., Haeckel, W., Witt, H. T. & Herrmann, R. G. *Nucleic Acids Res.* (in the press).
- Mühlenhoff, U. thesis, Technische Universität Berlin (1991).
- Döring, G., Bailey, J., Kreutz, W., Weikard, J. & Witt, H. T. *Naturwiss.* **55**, 219-220 (1968).
- Norris, J. R., Uphaus, R. A., Crespi, H. G. & Katz, J. J. *Proc. natn. Acad. Sci. U.S.A.* **68**, 625-629 (1971).
- Philips, K. D., Sato, V. L. & Sauer, K. *Biochemistry* **11**, 4591-4595 (1972).
- Golbeck, J. H. & Bryant, D. A. *Curr. Topics Bioenerg.* **16**, 83-177 (1991).
- Shuvalov, V. A., Nuijs, A. M., van Gorkom, H. J., Smit, H. W. J. & Duysens, L. M. N. *Biochim. biophys. Acta* **850**, 319-329 (1986).
- Biggins, J. & Mathis, P. *Biochemistry* **27**, 1494-1500 (1988).
- Brettel, K. *Biochim. biophys. Acta* **976**, 246-249 (1989).
- Wynn, R. M. & Malkin, R. *FEBS Lett.* **229**, 293-297 (1988).
- Mehari, T., Parett, K. G., Warren, P. V. & Golbeck, J. H. *Biochim. biophys. Acta* **1056**, 139-148 (1991).
- Ford, R. C., Picot, D. & Garavito, R. M. *EMBO J.* **6**, 1581-1586 (1987).
- Witt, I. *et al.* *FEBS Lett.* **221**, 260-264 (1987).
- Ford, R. C., Paupit, R. A. & Holzenburg, A. *FEBS Lett.* **238**, 385-389 (1988).
- Witt, I. *et al.* *Ber. Bunsenges. Phys. Chem.* **92**, 1503-1506 (1988).
- Shoham, G., Michaeli, D. & Nechushtai, R. *Current Research in Photosynthesis* vol. 2 (ed. Baltscheffsky, M.) 7555-7562, (Kluwer, Dordrecht, 1990).
- Almog, O., Shoham, G., Michaeli, D. & Nechushtai, R. *Proc. natn. Acad. Sci. U.S.A.* **88**, 5312-5316 (1991).
- Witt, H. T. *et al.* *Research in Photosynthesis 1*, (Proc. IX Int. cong. Photosynthesis, Nagoya, Japan (ed. Murata, N.) 521-528 (Kluwer, Dordrecht, 1992).
- McDermott, A. E. *et al.* *Biochemistry* **27**, 4013-4020 (1988).
- Scheller, H. V., Svendsen, I. & Lindberg-Møller, B. *J. biol. Chem.* **264**, 6929-6934 (1989).
- Petrouleas, V., Brand, J. J., Parett, K. G. & Golbeck, J. M. *Biochemistry* **28**, 8980-8983 (1989).
- Adman, E. T., Sieker, L. C. & Jensen, L. H. *J. biol. Chem.* **248**, 3987-3996 (1973).
- Oh-oka, H., Takahashi, Y., Kuriyama, K., Saeki, K. & Matsubara, H. *J. Biochem.* **103**, 962-968 (1988).
- Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S. & Dutton, P. L. *Nature* **355**, 796-802 (1992).
- Hiller, R. G., Bardin, A.-M. & Navedryk, E. *Biochim. biophys. Acta* **894**, 365-369 (1987).
- Kühlbrandt, W. & Wang, D. N. *Nature* **350**, 130-134 (1991).
- Haworth, P., Tapie, P., Arntzen, J. & Breton, J. *Biochim. biophys. Acta* **682**, 152-159 (1982).
- Matthews, B. W., Fenna, R. E., Bolognesi, M. C., Schmid, M. F. & Olson, J. M. *J. molec. Biol.* **131**, 259-285 (1979).
- Junge, W. & Witt, H. T. *Z. Naturforsch.* **23b**, 244-254 (1968).
- Rutherford, A. W. & Setif, P. *Biochim. biophys. Acta* **1019**, 128-132 (1990).
- Hippler, M., Ratajczak, R. & Haeckel, W. *FEBS Lett.* **250**, 280-284 (1989).
- Lüneberg, J. thesis, Technische Universität Berlin (1992).
- CCP4, The Co-operative Computing Project in Crystallography [SERC Daresbury Laboratory, Warrington, UK, 1986].
- Messerschmidt, A. & Pflugrath, J. W. *J. appl. Crystallogr.* **20**, 306-315 (1987).
- Wang, B. C. *Meth. Enzym.* **114**, 472-475 (1985).
- Leslie, A. G. W. *Acta crystallogr.* **A43**, 134-136 (1987).
- Jones, A. T. *J. appl. Crystallogr.* **11**, 268-272 (1978).



44. Yeates, T. O., Komyia, H., Rees, D. C., Allen, J. P. & Feher, G. *Proc. natn. Acad. Sci. U.S.A.* **84**, 6438–6442 (1987).  
 45. Boekema, E. J. *et al.* *FEBS Lett.* **217**, 283–286 (1987).  
 46. Ford, R. C. & Holzenburg, A. *EMBO J.* **7**, 2287–2293 (1988).  
 47. Ford, R. C., Hefti, A. & Engel, A. *EMBO J.* **9**, 3067–3075 (1990).  
 48. Rögner, M., Mühlenhoff, U., Boekema, E. J. & Witt, H. T. *Biochim. biophys. Acta* **1015**, 415–424 (1990).  
 49. Böttcher, B., Gräber, P. & Boekema, E. J. *Biochim. biophys. Acta* (in the press).  
 50. Hladik, J. & Sofrova, D. *Photosynth. Res.* **29**, 171–175 (1991).

51. Hladik, J., Pospisilova, L. & Sofrova, D. *Current Research in Photosynthesis* Vol. 2 (ed. M. Baltscheffsky) 579–582 (Kluwer, Dordrecht, 1990).  
 52. Jones, T. A., Zou, J.-Y., Cowan, S. W. & Kjeldgaard, N. *Acta crystallogr.* **A47**, 110–119 (1991).

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## Detection of supersoft X-ray emission from GQ Muscae nine years after a nova outburst

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GQ MUS (Nova Muscae 1983) was the first classical nova from which X-rays were detected during outburst<sup>1,2</sup>. Those Exosat observations were consistent with thermonuclear burning on the surface of a white dwarf emitting  $10^{37}$ – $10^{38}$  erg s<sup>-1</sup> at  $(3.0$ – $3.5) \times 10^5$  K or with shocked circumstellar material emitting  $10^{35}$  erg s<sup>-1</sup> by thermal bremsstrahlung at  $10^7$  K. Here we report the detection by the Rosat satellite<sup>3</sup> of GQ Mus as a very soft black-body-like source. If the observed X-ray flux is being radiated at the Eddington luminosity ( $10^{38}$  erg s<sup>-1</sup>) from a one-solar-mass white dwarf, its effective temperature must be  $\sim 3.5 \times 10^5$  K. We conclude that the white dwarf is burning hydrogen-rich material near its surface. GQ Mus is, however, the only one of 26 recent novae detected in the all-sky Rosat survey; this suggests that either most novae eject all their accreted material during outburst, or GQ Mus is now burning recently accreted material. GQ Mus appears identical to the supersoft X-ray sources CAL83, CAL87 and RX J0527.8–6954 (ref. 4), lending support to the suggestion that these sources are white dwarfs accreting and burning material from a companion<sup>5</sup>.

The classical nova outburst is commonly assumed to occur on the white dwarf component of a close binary system (cataclysmic variable). The white dwarf accretes material from the companion star and a thick electron-degenerate envelope is gradually constructed on the surface of the white dwarf, leading eventually to a thermonuclear runaway and an explosion<sup>6</sup>. As a direct result,  $\sim 10^{-6}$  to  $10^{-4}$  solar masses ( $M_{\odot}$ ) of material is ejected. But hydrodynamic studies of the classical nova outburst<sup>6,7</sup> find that only a fraction of the envelope is ejected by the initial explosion. The remaining material quickly returns to quasistatic equilibrium and a phase of steady nuclear burning and constant bolometric luminosity is initiated, during which strong winds may also eject the envelope<sup>8–11</sup>. As the envelope is lost, the photosphere will slowly recede within the expanding material causing the effective temperature to increase and the peak of the emitted radiation to move into the soft X-ray region. The detection of this constant bolometric luminosity phase, which should last until the envelope is exhausted, has been one of the challenges of novae observations.

As a part of a programme to monitor the soft X-ray emission of recent novae, GQ Mus was observed with the Rosat PSPC during 25–26 February 1992 with an effective exposure time of

5,840 s. Descriptions of the satellite and the X-ray telescope can be found in ref. 3. The net count rate from GQ Mus was  $0.108 \pm 0.004$  counts s<sup>-1</sup> in the 0.1–0.8-keV range and there were nearly no source counts above 0.8 keV. A search for possible modulation at the known orbital period of  $85.5 \pm 0.4$  min (ref. 12) showed no signal above the statistical fluctuations of  $\sim 30\%$ . There is a weak hint (80% confidence) that the source is variable over the observation span.

The remarkable aspect of the background-subtracted source counts from GQ Mus was the softness of the raw spectrum. As shown in Table 1, the best spectral fit to the data ( $\chi^2 = 18.5$  for 16 degrees of freedom) was a black body with number density of hydrogen  $N_H = 3.4 \times 10^{21}$  cm<sup>-2</sup>,  $kT = 23$  eV and a bolometric flux of  $3.7 \times 10^{-6}$  erg cm<sup>-2</sup> s<sup>-1</sup>. Figure 1 shows the count rate and incident flux fitted to the data with the parameters listed above. The  $N_H$  parameter is close to the value  $(3 \pm 1) \times 10^{21}$  cm<sup>-2</sup> (ref. 2) derived from the  $E_{B-V}$  value of  $0.45 \pm 0.15$  (ref. 13). However, the bolometric flux is about a factor 100 too high for the Eddington limit of  $\sim 10^{38}$  erg s<sup>-1</sup> for a white dwarf of  $1 M_{\odot}$  at a distance of  $4.7 \pm 1.5$  kpc (ref. 13). On the other hand, within the 2 and  $3\sigma$  error limits, the black-body fit does allow higher temperatures and lower fluxes. In Fig. 2 we plot the 1, 2 and  $3\sigma$  contours in the black-body temperature/bolometric-flux plane where the  $N_H$  value has been allowed to vary between  $2 \times 10^{21}$  and  $4 \times 10^{21}$  cm<sup>-2</sup>. As can be seen in the figure, the 2

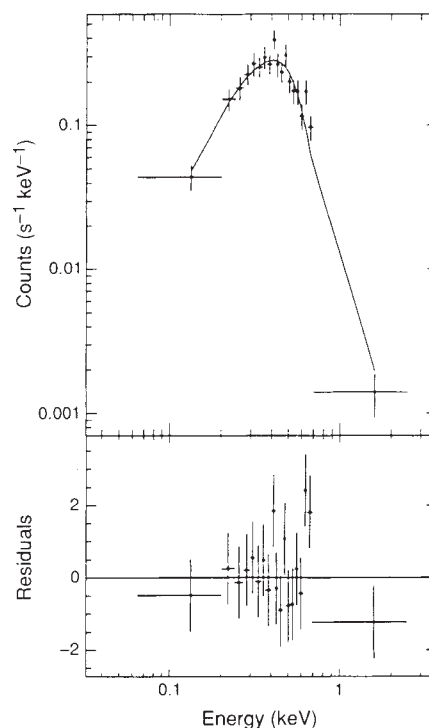


FIG. 1 Energy spectrum of GQ Mus. The data points are the observed counts s<sup>-1</sup> keV<sup>-1</sup> and the solid line is the best fitting model of a black body with  $N_H = 3.4 \times 10^{21}$ ,  $kT = 23$  eV and bolometric flux  $f_{bol} = 3.7 \times 10^{-6}$  cm<sup>-2</sup> s<sup>-1</sup>. The lower panel shows the residuals from the model fit in standard deviation.

and  $3\sigma$  contours approach the Eddington luminosity  $L_{\text{Edd}}$  for a  $\sim 1 M_{\odot}$  white dwarf and a distance of  $4.7 \pm 1.5$  kpc. The implied effective temperature  $T_{\text{eff}}$  of the white dwarf is limited to the range 28.8–30.9 eV ( $\sim 3.5 \times 10^5$  K). The black-body radius that can emit the observed flux in this temperature range is  $(2.9\text{--}3.4) \times 10^9$  cm. Comparable white-dwarf parameters have been indirectly inferred from the optical observations of coronal lines<sup>14,15</sup>. A further check on the fitted spectrum was obtained from contemporaneous ultraviolet observations with the International Ultraviolet Explorer satellite on 16 April 1992. The dereddened ultraviolet luminosity for the wavelength region 1,150 Å to 2,000 Å, and a distance of 4.7 kpc, was  $1.7 \times 10^{35}$  erg s<sup>-1</sup>. For  $T_{\text{eff}} = 3.5 \times 10^5$ , the bolometric luminosity corresponding to the ultraviolet data is  $1.2 \times 10^{38}$  erg s<sup>-1</sup>, in good agreement with the X-ray fit obtained above.

The energy source for the observed luminosity of GQ Mus must be hydrogen burning, which is  $\sim 30$  times more efficient than gravitational energy release from accretion onto the white dwarf. As the energy yield per unit mass by hydrogen burning (for 0.7 hydrogen mass fraction) is  $\sim 4.5 \times 10^{13}$  erg g<sup>-1</sup>, the present X-ray luminosity implies that  $\dot{M} \geq 10^{-7} M_{\odot} \text{ yr}^{-1}$  is currently being burned. In principle, this is in agreement with the constant-bolometric-luminosity model which implies that a considerable fraction of the envelope still remains on the white dwarf. However, GQ Mus seems to be an exceptional nova; during the Rosat all-sky survey it was the only nova detected among 26 novae which have undergone an outburst within the past 10 years<sup>16</sup>. In addition, it is the only well studied nova to show very strong [Fe X] (ref. 15) and [Fe XI] (J. Krautter, unpublished observations) in emission, implying a very hot underlying source. The Rosat survey results force us to conclude that steady nuclear burning of the remaining hydrogen-rich envelope occurs at most in a few per cent of the classical novae and therefore most novae must eject their envelopes on a very rapid timescale. The two recent novae detected by Rosat during their early outburst stage, Nova Hercules 1991 (ref. 17) and Nova Cygni 1992 (ref. 18), both show hard spectra with luminosities below  $10^{-2} \times L_{\text{Edd}}$ . It is likely that the X-ray emission in these two novae involves shock heating of the ejected material and is different from the source of X-rays in GQ Mus.

Alternatively, we suggest that the source of the hydrogen-rich

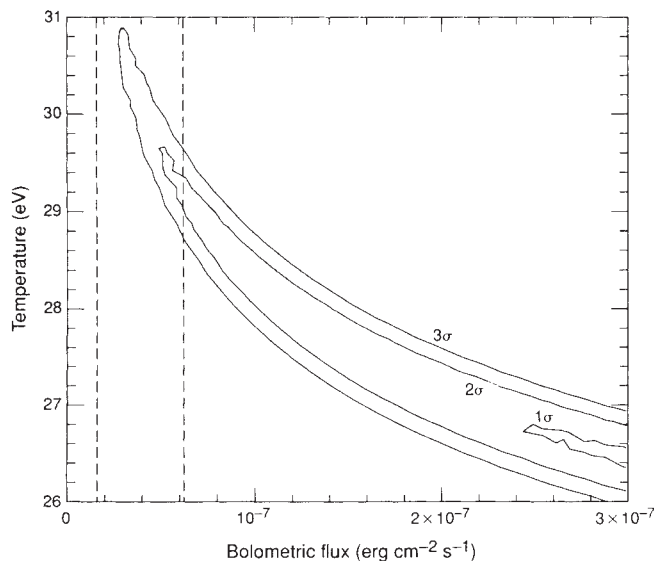


FIG. 2 The 1, 2 and  $3\sigma$  contours of the black-body model fit in the effective-temperature-bolometric-flux plane. The Eddington limit flux for  $L = 10^{38}$  erg s<sup>-1</sup> and a distance of  $4.7 \pm 1.5$  kpc is indicated by vertical dashed lines.

TABLE 1 Best black-body model fit to observed GQ Mus energy spectrum

| Model                                                                                         | $N_{\text{H}}$ (cm <sup>-2</sup> ) | $kT$ (eV) | Bolometric flux<br>(erg cm <sup>-2</sup> s <sup>-1</sup> ) |
|-----------------------------------------------------------------------------------------------|------------------------------------|-----------|------------------------------------------------------------|
| Free fit                                                                                      | $3.4 \times 10^{21}$               | 23        | $3.7 \times 10^{-6}$                                       |
| Flux limited to<br>$L = 10^{38}$ erg s <sup>-1</sup> at<br>a distance of<br>$4.7 \pm 1.5$ kpc | $(1\text{--}4) \times 10^{21}$     | 28.8–30.9 | $(1.6\text{--}6.2) \times 10^{-8}$                         |

fuel of GQ Mus may be fresh material, currently being accreted from the companion at a rate of  $\sim 10^{-7} M_{\odot} \text{ yr}^{-1}$ . With the orbital period of 85.5 min (ref. 12), the companion of GQ Mus has to have a mass of  $\leq 0.2 M_{\odot}$  if we impose the condition that it is a main-sequence star overflowing its Roche lobe. The irradiation of the secondary will be an important factor in determining the required high mass-transfer rate. It has been suggested that the irradiation-induced mass-transfer rate is inversely proportional to both the secondary mass and the orbital separation<sup>19</sup>, which are both unusually small in the case of GQ Mus. Regardless of the details, if such a high  $\dot{M}$  is occurring, it should cause the orbital separation to increase on a timescale much shorter than the timescales of gravitational radiation or magnetic braking<sup>20,21</sup>. Consequently, for conservative mass transfer, the orbital period should increase at a rate  $\dot{P}_{\text{orb}}/P_{\text{orb}} \approx 10^{-6} \text{ yr}^{-1}$  and lead to a phase delay of  $20t^2$  seconds (where  $t$  is the elapsed time between observations in years), which is an observable number. The duration of this high rate of mass transfer would then be determined by the delicate balance between the decrease of the orbital separation and the adiabatic expansion of the fully convective secondary, which is hard to predict without detailed calculations.

Our results for the spectrum and luminosity of GQ Mus are strikingly similar to those of the supersoft Large Magellanic Cloud (LMC) X-ray sources CAL83, CAL87 and RX J0527.8–6954 (ref. 4). Although it has been proposed that these are a new class of accreting neutron-star binaries<sup>4,22</sup>, van den Heuvel *et al.*<sup>5</sup> have suggested that they can be explained by steady nuclear burning of hydrogen accreted onto white dwarfs with main-sequence companions in the mass range  $1.5\text{--}2.0 M_{\odot}$ . The larger secondary mass ensures that the mass transfer is unstable and proceeds on the thermal timescale of the secondary,  $\sim 10^7$  yr. These Rosat observations of GQ Mus have shown that the white dwarf in a post-nova cataclysmic variable can appear as a steady nuclear-burning supersoft X-ray source over timescales longer than 10 years. Can GQ Mus-type novae explain the observed supersoft LMC sources? Statistically, the observed LMC novae rate of  $2 \text{ yr}^{-1}$  (ref. 23), multiplied by the estimated fraction of novae that behave like GQ Mus ( $\sim 1/26$ ) and by a lifetime of  $\geq 10$  yr, yields  $\geq 1$  such sources observable any time; within the uncertainties, this number is compatible with the three sources observed by Rosat. A more complete search might triple the number of supersoft LMC sources, which in turn would require the lifetime of the steady nuclear burning phase to be  $\sim 100$  yr. It is not surprising that none of the supersoft LMC sources have been identified with historical novae, as the outburst could have occurred 100 years ago. Even more recent LMC novae may have gone undetected, because a systematic search has been going on for only two decades. On the other hand, the 1.04-day period of Cal 83 (ref. 24) implies that the binary separation may be too large for the irradiation-induced mass-transfer mechanism to work, thus suggesting that at least some of the supersoft sources may be steady nuclear-burning white dwarfs accreting from high-mass companions<sup>5</sup>.

Our results establish a connection between novae phenomena and the newly discovered supersoft X-ray sources. This connection may aid our understanding of the evolution of white dwarfs in close binaries from their early stages to possible accretion-induced collapse to a neutron star. It will be interesting to follow



the developments of GQ Mus, both in its X-ray light curve and in the variation of its orbital period. □

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1. Ögelman, H., Beuermann, K. & Krautter, J. *Astrophys. J.* **287**, L31–L34 (1984).
2. Ögelman, H., Krautter, J. & Beuermann, K. *Astr. Astrophys.* **177**, 110–116 (1987).
3. Trümper, J. *et al. Adv. Space Res.* **2**, 241–249 (1983).
4. Greiner, J., Hasinger, G. & Kahabka, P. *Astr. Astrophys.* **246**, L17–L20 (1991).
5. van den Heuvel, E. P. J., Bhattacharya, D., Nomoto, K. & Rappaport, S. A. *Astr. Astrophys.* **262**, 97–105 (1992).
6. Starrfield, S., Truran, J. W., Sparks, W. M. & Kutter, G. S. *Astrophys. J.* **176**, 169–173 (1974).
7. Prialnik, D., Shara, M. M. & Shaviv, G. *Astr. Astrophys.* **62**, 339–348 (1978).
8. MacDonald, J., Fujimoto, M. Y. & Truran, J. W. *Astrophys. J.* **294**, 263–273 (1985).
9. Starrfield, S., Truran, J. W., Sparks, W. & Krautter, J. in *Extreme Ultraviolet Astronomy* (eds Malina, R. & Bowyer, S.) 168–176 (Pergamon: New York, 1991).
10. Kato, M. *Publ. Astr. Soc. Japan* **35**, 507–519 (1983).
11. Orio, M., Trussoni, E. & Ögelman, H. *Astr. Astrophys.* **257**, 548–556 (1992).
12. Diaz, M. D. & Steiner, J. E. *Astrophys. J.* **239**, L41–L43 (1989).
13. Krautter, J. *et al. Astr. Astrophys.* **137**, 307–326 (1984).
14. Diaz, M. P., Williams, R. E., Phillips, M. M. & Steiner, J. E. in *Viña del Mar Workshop on Cataclysmic Variable Stars* (ed. Vogt, N.) *Astr. Soc. Pac. Conf. Series* **29**, 362–368 (1992).
15. Krautter, J. & Williams, R. E. *Astrophys. J.* **341**, 968–973 (1989).
16. Orio, M. *et al. Proc. 1992 COSPAR Symp.* (in the press).
17. Lloyd, H. M. *et al. Nature* **356**, 222–224 (1992).
18. Krautter, J., Ögelman, H. & Starrfield, S. *IAU Circ. no.* 5550.
19. Kovetz, A., Prialnik, D. & Shara, M. M. *Astrophys. J.* **325**, 828–836 (1988).
20. Landau, L. D., Lifshitz, E. M. *The Classical Theory of Fields*, 325 (New York: Pergamon, 1971).
21. Verbunt, F. & Zwaan, C. *Astr. Astrophys.* **100**, L7–L9 (1981).
22. Trümper, J. *et al. Nature* **349**, 579–583 (1991).
23. Capaccioli, M., Della Valle, M., D'Onofrio, M. & Rosino, L. *Astrophys. J.* **360**, 63–67 (1990).
24. Smale, A. P. *et al. Mon. Not. R. Astr. Soc.* **233**, 51–63 (1988).

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## Capillarity-induced filling of carbon nanotubes

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THE recent discovery<sup>1</sup> and bulk synthesis<sup>2</sup> of nanometre-scale carbon tubules has led to much speculation about possible uses of these graphitic structures<sup>3–5</sup>. Broughton and Pederson predicted on the basis of computer simulations that open nanotubes may be filled with liquid by capillary suction<sup>6</sup>. Here we describe experiments in which annealing of the tubules in the presence of liquid lead results in opening of the capped tube ends and subsequent filling of the tubes with molten material through capillary action. The nanotubes thus act as moulds for the fabrication of (possibly metallic) wires, some of which are less than two nanometres in diameter.

We synthesized carbon nanotubes as bulk cylindrical deposits, according to the method of ref. 2. The core of the deposit, which contains the nanotubes, was sonicated in ethanol and a drop of the solution was dried over a perforated carbon grid. Lead particles were then deposited onto the tubes following electron-

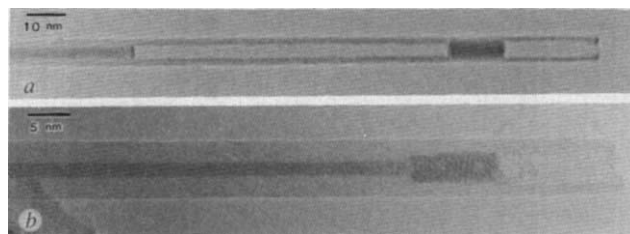


FIG. 2 Carbon nanotubes with the ends open (right side) after annealing (see text). *a*, Solid material trapped in two regions of the tube is seen as regions of dark contrast. *b*, Open-ended tube with filling, taken at higher magnification. The diameter of the filling varies with the size of the inside hollow of the tube.

beam evaporation of pure lead under a vacuum of  $10^{-6}$  torr. A typical nanotube after evaporation of lead particles is shown in Fig. 1; the size of the lead particles on the surface of the tubules is of the order of 1–15 nm. Lead particles were also often deposited on the closed caps of the tubes. The grid containing the sample was then annealed in an oven in air for about 30 min, keeping the temperature at approximately 400 °C, which is above the melting point of lead.

Examination of the annealed sample by transmission electron microscopy (TEM) showed that the interior of some of the nanotubes had become filled with solid material, which shows up as regions of darker contrast in the images (Figs 2 and 3). Figure 2*a*, taken at lower magnification, shows trapping of the material at two regions inside the tube. The tube is open at the right end, through which molten material appears to have been sucked in. We have observed the same filling effect after strong electron irradiation of nanotube tips that are decorated with metal particles and which have become opened during annealing. Under strong irradiation, the material decorating the open tube tips melts and enters the inside channel. We have found that filling of the tubes does not readily occur when the annealing is performed in vacuum for the same duration at similar temperatures; the tube tips remain closed. This suggests that opening of the tips involves a chemical reaction between the metal, oxygen and the carbon that makes up the tubes. The capped portions of the outer and inner tubes are apparently attacked

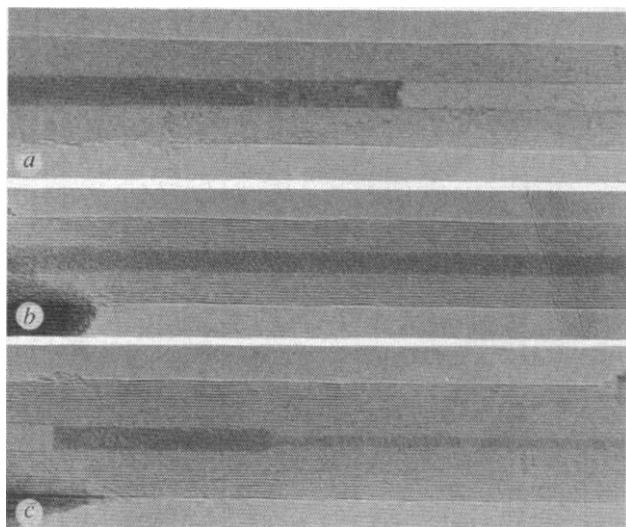


FIG. 3 High-resolution TEM images of carbon nanotubes filled with solid (dark contrast) for tens of nanometres. The original shape and condition of the tube is retained with very little damage on the surface. The distance between parallel fringes is 0.34 nm.

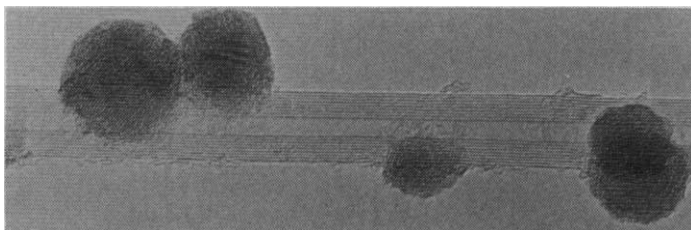


FIG. 1 High-resolution TEM image of a typical carbon nanotube with lead particles deposited on the surface. The parallel fringes of the carbon tube are 0.34 nm apart.

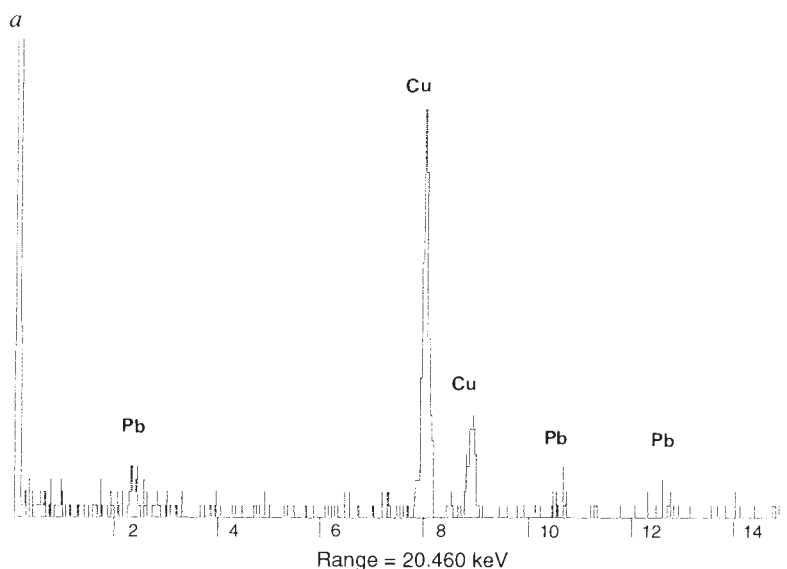
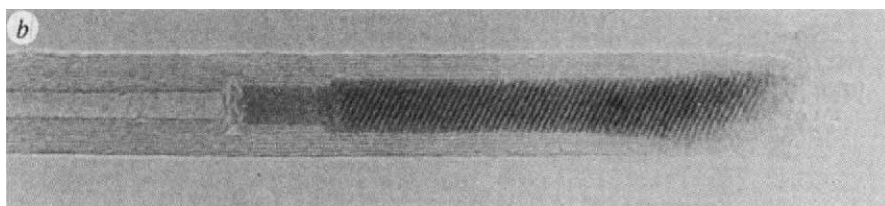


FIG. 4 *a*, Energy-dispersive X-ray data from filled tubes, showing the presence of  $M$ ,  $L_{\alpha}$  and  $L_{\beta}$  peaks from lead. The large peaks from copper ( $K_{\alpha}$  and  $K_{\beta}$ ) are due to the copper grid that supports the specimen. *b*, High-resolution TEM image of a crystalline portion of filling material near the open end of a wide tube. The distance between the concentric carbon layers in the tube is 0.34 nm. The spacing between lattice fringes of the filling does not match that of known lead compounds.



and destroyed during the heating in air, following which molten material is drawn inside by strong capillary forces. Another open-ended and filled tube is shown in Fig. 2*b*, taken at higher magnification. Except for the tips, the tubes are undamaged, with perfect concentric layers. Presumably the presence of pentagonal  $C_5$  rings at the tips make them reactive relative to the hexagonal network of the cylindrical tube surfaces.

Figure 3*a–c* show tubes filled with a solid non-carbon phase with the filling extending up to many tens of nanometres. Once the molten material enters, strong capillary forces<sup>6</sup> appear to draw it into thin wires. The meniscus of the indrawn material is clearly seen in Fig. 3*a*. The filling is seen to wet the inside surface at least partially—the contact angle is usually above about 60°. The diameter of the filling changes with the size of the interior hollow, as seen in Fig. 3*c* (the diameter of the inside channels can change because of capping and terminations of inside layers<sup>7</sup>). In effect the interior of the nanotube acts as a mould for fabrication of a 'nanowire'. In Fig. 3*c*, the thinner wire has a diameter of less than 1.5 nm.

The structure of the filling, especially in narrow channels (Fig. 3), appears to be disordered in most cases. We cannot yet determine whether the filling is pure lead metal or a compound formed by reaction of lead with carbon and oxygen. We have identified the presence of lead inside the channels with energy-dispersive X-ray analysis (Fig. 4*a*) and electron energy-loss spectroscopy, but we found no signal corresponding to oxygen. The TEM contrast of the filling is not as strong as for bulk metals, so the material may be quite porous. Near the open ends of wider tubes, we have seen lattice fringes from the filling, indicating a crystalline structure (Fig. 4*b*); but the fringe spacing does not match with those from any known binary or ternary phase of lead compounds with oxygen and carbon or with that of pure crystalline lead metal. In very narrow channels the structure seems to correspond to a disordered glassy phase. The annealing temperature is not high enough to melt the known stable oxide of lead (PbO), which might be formed under these conditions. The solidification of molten material in such con-

strained geometries could conceivably be very different from that in the bulk, and new phases might be formed.

In the sample we have studied, less than ~1% of the tubes were filled. This could be because the metal was not in contact with all of the tube tips during annealing. With larger amount of the metal present, a more favourable distribution of metal particles and longer annealing times, the filled fraction might be much higher. The number of lead particles on the tube surfaces decreased markedly after annealing, suggesting strong dewetting of the tubes by the particles. The tips of almost all of the filled tubes seemed to be damaged. We observed no intercalation of the indrawn material between the carbon layers.

The modification of carbon nanotubes into composite nanofibres by filling them in this way opens up several possibilities for potential uses. Filled tubes should be easy to separate from empty tubes by separation techniques that depend on density. The mechanical properties of the modified fibres could be very different from the original carbon material. Encapsulation might have interesting effects on the properties (such as superconductivity or magnetism) of the interior phase (lead metal is a superconductor at ~7.2 K). The fabrication of nanowires using carbon nanotubes as the template provides a new approach to nano-fabrication. The dynamics of wetting inside the tubes might be studied by *in situ* microscopic observations. The high reactivity of the tube caps, where five-membered carbon rings are present, compared with the cylindrical tube surfaces remains to be understood. □

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1. Iijima, S. *Nature* **354**, 56–58 (1991).
2. Ebbesen, T. W. & Ajayan, P. M. *Nature* **358**, 220–222 (1992).
3. Calvert, P. *Nature* **357**, 365–366 (1992).
4. Mintmire, J. W., Dunlap, B. I. & White, C. T. *Phys. Rev. Lett.* **68**, 631–634 (1992).
5. Hamada, N., Sawada, S. & Oshiyama, A. *Phys. Rev. Lett.* **68**, 1579–1581 (1992).
6. Pederson, M. R. & Broughton, J. Q. *Phys. Rev. Lett.* **69**, 2689–2692 (1992).
7. Iijima, S., Ajayan, P. M. & Ichihashi, T. *Phys. Rev. Lett.* **69**, 3100–3113 (1992).

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# Absence of evidence for greenhouse warming over the Arctic Ocean in the past 40 years

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ATMOSPHERIC general circulation models predict enhanced greenhouse warming at high latitudes<sup>1</sup> owing to positive feedbacks between air temperature, ice extent and surface albedo<sup>2-4</sup>. Previous analyses of Arctic temperature trends have been restricted to land-based measurements on the periphery of the Arctic Ocean<sup>5,6</sup>. Here we present temperatures measured in the lower troposphere over the Arctic Ocean during the period 1950–90. We have analysed more than 27,000 temperature profiles, measured by radiosonde at Russian drifting ice stations and by dropsonde from US 'Ptar-migan' weather reconnaissance aircraft, for trends as a function of season and altitude. Most of the trends are not statistically significant. In particular, we do not observe the large surface warming trends predicted by models; indeed, we detect significant surface cooling trends over the western Arctic Ocean during winter and autumn. This discrepancy suggests that present climate models do not adequately incorporate the physical processes that affect the polar regions.

According to general circulation model (GCM) studies, both with and without coupled ocean model components, high-latitude warming in response to increasing greenhouse gas concentrations should occur rapidly both at the surface and at higher elevations in the troposphere<sup>1</sup>. In one simulation<sup>7</sup>, Arctic surface air temperatures for the 1980s were predicted to increase by 2 °C relative to 1958 levels. There is marked disagreement, however, among simulations of the present-day Arctic climate. Walsh and Crane<sup>8</sup> found significant differences among five GCMs in reproducing basic meteorological fields, including sea-level pressure and surface air temperature. As the causes for the large intra- and interannual differences in the Arctic

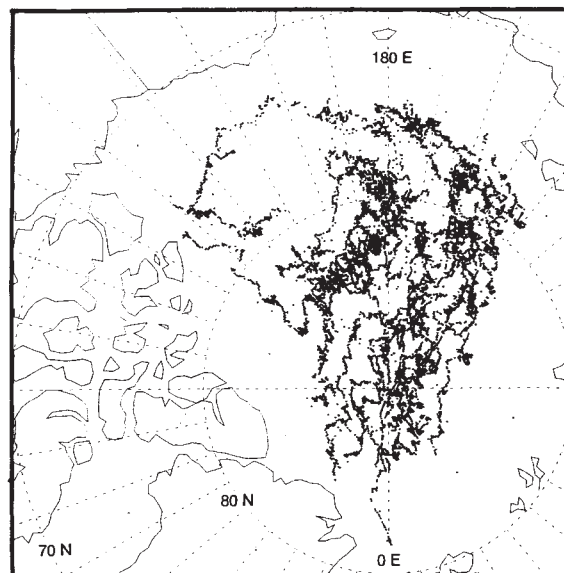


FIG. 1 Locations of 16,850 temperature profiles measured by radiosonde at Russian drifting ice stations during 1954–1990. The stations drift with the prevailing winds and surface currents in the Arctic Ocean, typically moving ~100 km each month. Taken together, the measurements provide reasonably complete coverage over the Arctic ice cap. At any given time, however, data are only available at locations along the drift path.

circulation and hence tropospheric temperatures are still poorly understood<sup>9</sup>, it is not surprising that the interannual variability in tropospheric temperatures may be poorly simulated. In addition, many GCMs lack thermodynamic sea-ice model components which are obviously of great importance in the polar regions.

As predicted by theory, Arctic surface temperatures have shown accelerated warming trends during the past century<sup>5,6</sup>. Temperatures above the surface, however, have shown little evidence of enhanced warming during the past several decades<sup>10–12</sup>. Tropospheric cooling has even been detected in some regions of the Arctic<sup>13</sup>. So far analyses of Arctic temperature trends have focused on measurements at land-based weather stations located along the periphery of the nearly uninhabited Arctic Ocean. Here we analyse temperature measurements made over the Arctic Ocean during the period 1950–1990.

Two independent meteorological databases were assembled for analysis. The first consists of 16,850 lower tropospheric temperature profiles measured at the 'North Pole' series of

TABLE 1 Temperature trends over central and western Arctic Ocean

| Season               | Surface      |          |          | 850 hPa     |          |          | 700 hPa     |          |          | 850-700 hPa layer |          |          |
|----------------------|--------------|----------|----------|-------------|----------|----------|-------------|----------|----------|-------------------|----------|----------|
|                      | Trend        | <i>N</i> | <i>s</i> | Trend       | <i>N</i> | <i>s</i> | Trend       | <i>N</i> | <i>s</i> | Trend             | <i>N</i> | <i>s</i> |
| Central Arctic Ocean |              |          |          |             |          |          |             |          |          |                   |          |          |
| Winter               | 0.81         | 30       | 0.30     | <b>3.41</b> | 30       | 0.98     | 2.14        | 30       | 0.94     | <b>3.30</b>       | 30       | 0.99     |
| Spring               | 0.02         | 26       | 0.00     | 1.06        | 27       | 0.36     | 2.33        | 26       | 0.78     | 3.26              | 26       | 0.91     |
| Summer               | 0.41         | 26       | 0.62     | 0.81        | 26       | 0.60     | 0.12        | 26       | 0.09     | 1.31              | 26       | 0.80     |
| Autumn               | −2.50        | 29       | 0.79     | −0.02       | 29       | 0.01     | −0.07       | 29       | 0.04     | 0.71              | 29       | 0.42     |
| Western Arctic Ocean |              |          |          |             |          |          |             |          |          |                   |          |          |
| Winter               | <b>−4.40</b> | 20       | 0.97     | <b>3.42</b> | 20       | 0.97     | <b>2.54</b> | 20       | 0.96     | <b>3.74</b>       | 20       | 0.99     |
| Spring               | 1.56         | 20       | 0.27     | 2.39        | 20       | 0.54     | 2.51        | 20       | 0.65     | 2.45              | 20       | 0.59     |
| Summer               | −0.23        | 23       | 0.19     | −0.49       | 23       | 0.38     | −0.21       | 22       | 0.20     | 0.18              | 22       | 0.14     |
| Autumn               | <b>−4.99</b> | 21       | 0.96     | −1.14       | 21       | 0.48     | −1.34       | 21       | 0.66     | −1.06             | 21       | 0.51     |

Temperature trends (°C per 40 yr) over the central and western Arctic Ocean, along with the length of the data record in years (N) and the confidence level (s). Bold entries are significant at the 95% confidence level or greater. Note that most trends are not significant, and that significant warming in surface air temperatures is not observed.

TABLE 2 Temperature trends over Arctic Ocean

| Season | Trend        | Surface |      | Trend       | 850 hPa |      | Trend | 700 hPa |      | Trend       | 850–700 hPa layer |      |
|--------|--------------|---------|------|-------------|---------|------|-------|---------|------|-------------|-------------------|------|
|        |              | N       | s    |             | N       | s    |       | N       | s    |             | N                 | s    |
| Winter | <b>−2.44</b> | 38      | 0.95 | <b>2.30</b> | 38      | 0.97 | 1.74  | 38      | 0.94 | <b>2.77</b> | 38                | 0.99 |
| Spring | 0.70         | 36      | 0.19 | 2.08        | 36      | 0.77 | 2.12  | 36      | 0.84 | 2.66        | 36                | 0.90 |
| Summer | 0.00         | 38      | 0.01 | −0.11       | 38      | 0.11 | −0.21 | 38      | 0.24 | 0.64        | 38                | 0.59 |
| Autumn | <b>−4.14</b> | 37      | 0.99 | −1.07       | 37      | 0.77 | −1.19 | 37      | 0.85 | −0.62       | 37                | 0.52 |

Temperature trends (°C per 40 yr) over the Arctic Ocean, along with the length of the data record in years (*N*) and the confidence level (*s*). Bold entries are significant at the 95% confidence level or greater. Results are shown for all available measurements. Most trends are not significant, and significant cooling in surface air temperatures is observed during winter and autumn.

Russian drifting ice stations during the period 1954–1990 (Fig. 1). Temperature profiles were measured 1–2 times daily by radiosondes (balloon-borne meteorological instrument packages). Portions of this database have been used previously in investigations of the thermal structure over the Arctic Ocean<sup>14–16</sup>, convection heights above leads<sup>17</sup> and Arctic temperature fluctuations<sup>18</sup>. The second database contains 10,326 dropsonde temperature soundings measured during the 'Ptarmigan' air weather reconnaissance missions conducted by the United States Air Force during the period 1950–1961 (ref. 19) (Fig. 2). The dropsondes, meteorological instrument packages carried by parachutes, provided temperature profiles throughout the lower troposphere.

Temperatures at the surface, the 850 hPa (~1.4 km) and 700 hPa (~2.8 km) pressure altitudes, and mean virtual temperatures within the 850–700 hPa layer were extracted from each profile and subjected to quality-control procedures<sup>20,21</sup>. Virtual temperature is the temperature that dry air would have if it had the same density as moist air at the same pressure. Mean virtual temperature is a convenient quantity for monitoring climate change, as it characterizes an atmospheric layer and may be calculated from pressure and height data. It is a useful surrogate for temperature in the Arctic where moisture levels are low. Seasonal mean temperatures for each level or layer, based on a minimum requirement of 10 values per season, were calculated with seasons defined as winter (December–February), spring (March–May), summer (June–August) and autumn (September–November). Trends were computed by linear regression on seasonal anomalies for the 41-year period 1950–1990.

Statistical significance levels (for rejecting the null hypothesis that there is no trend) were determined through a bootstrap procedure<sup>22</sup> as follows. The anomaly time series were first detrended by subtracting the linear regression values from the *N* anomalies, where *N* is the number of points (years) in the time series. Bootstrap anomaly series were then obtained by drawing *N* values at random from the detrended anomaly series, allowing values to be drawn multiple times. The trend given by the linear regression was then added to the bootstrap anomaly series, and a new trend was subsequently calculated. This procedure was repeated 10<sup>4</sup> times for each season, level and layer, yielding gaussian frequency distributions of simulated trends with mean  $\mu$  and standard deviation  $\sigma$ . The significance level, the probability that the trend is significantly different from zero, was obtained by determining the number of standard deviations between  $\mu$  and zero ( $\mu/\sigma$ ) and integrating over the range  $\mu \pm \mu/\sigma$ .

Our analysis focused on two regions (Fig. 2): the 'central Arctic Ocean' (82–90° N) and the 'Western Arctic Ocean' (73–82° N and 90–180° W). These regions were defined through an iterative process in which we tried to optimize the number of annual observations within each region, while keeping the regions as small as possible. Possible sources of bias arising from the spatial and temporal inhomogeneity of the database will be discussed below.

The calculated temperature trends are shown in Table 1. For the central and western Arctic Ocean respectively, 26–30 and 20–23 years are represented in the anomaly time series analysed.

Most of the trends are not significant at the 95% confidence level. Winter trends are mostly positive and significant, particularly at above-surface levels in the western Arctic Ocean, although the large surface warming predicted by GCMs is not found in either region. Rather, a large, statistically significant surface cooling (negative) trend is found in the western Arctic during winter and autumn.

We conducted tests to assess the sensitivity of the computed trends to three possible sources of bias arising from the spatial and temporal non-uniformity of the data base: (1) the large degree of spatial anisotropy in the 'Ptarmigan' soundings (Fig. 2); (2) the temporal non-uniformity of the data base, as more observations were available for analysis during the years 1954–1961 (when both 'Ptarmigan' and Russian ice station soundings were available) than during other years; and (3) the selection of analysis regions. The first two sources of possible bias were investigated by excluding the 'Ptarmigan' soundings from the trend analyses. Without this portion of the database, the measurements were considerably more uniform in both space (Fig. 1) and time. The results were similar to those shown in Table 1. We addressed the third possible source of bias by computing trends for the entire Arctic Ocean using all available soundings including those outside the central and western Arctic Ocean analysis regions (Figs 1, 2). The results of this latter analysis (Table 2) are again similar to those shown in Table 1.

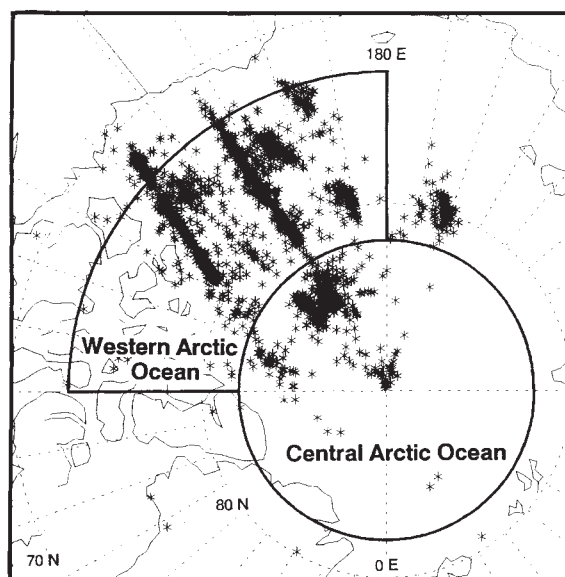


FIG. 2 Locations of 10,326 temperature profiles measured by dropsonde from U.S. Ptarmigan weather reconnaissance aircraft during 1950–1961. The aircraft typically flew diamond-shaped patterns extending from central Alaska to the North Pole. Portions of the flight paths are visible as 'streaks' of dropsonde positions. Drops were often made at pre-determined locations, with as many as 500 soundings made at specific points. More than 100 soundings were made at the North Pole. The 'Central Arctic Ocean' and 'Western Arctic Ocean' analysis regions are identified.



Few of the trends are significant, large surface warming trends are not observed, and like the Western Arctic Ocean analysis in Table 1, significant surface cooling trends are found during winter and autumn. Significant warming trends are observed at the 850 hPa level and the 850–700 hPa layer during winter, in agreement with GCM simulations, but the surface trend is negative. The trends presented in Table 2 are more representative temporally, as they use 36–38 years out of a possible 41, as compared with 20–30 years in the regional analysis (Table 1). On the basis of these tests, we feel that any possible bias introduced by the non-uniform database is small.

The lack of widespread significant warming trends leads us

to conclude that there is no strong evidence to support model simulations of greenhouse warming over the Arctic Ocean for the period 1950–1990. Our results, combined with the inconsistent performance of model simulations of Arctic climate<sup>6</sup>, indicate a need to understand better the physical processes that affect the polar regions, especially atmosphere–ice–ocean interactions, ocean heat transfer and cloud radiative effects, and to incorporate thermodynamic sea-ice components into future models. We consider the retrieval of temperature profiles from satellite sounding instruments to be an important means of further resolving the spatial and temporal gradients in Arctic air temperatures. □

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- Houghton, J. T. (ed.) *Climate Change: The IPCC Scientific Assessment* (Cambridge Univ. Press, Cambridge, 1990).
- Budyko, M. I. *Tellus* **21**, 611–619 (1969).
- Sellers, W. D. *J. appl. Meteorol.* **8**, 392–400 (1969).
- Ingram, W. J., Wilson, C. A. & Mitchell, F. J. B. *J. geophys. Res.* **94**, 8609–8622 (1989).
- Kelly, P. M., Jones, P. D., Sear, C. B., Cherry, B. S. G. & Tavakol, R. K. *Mon. Weath. Rev.* **110**, 71–83 (1982).
- Hansen, J. & Lebedeff, S. *J. geophys. Res.* **92**, 13345–13372 (1987).
- Hansen, J. et al. *J. geophys. Res.* **93**, 9341–9364 (1988).
- Walsh, J. E. & Crane, R. G. *Geophys. Res. Lett.* **19**, 29–32 (1992).
- Walsh, J. E. & Chapman, W. L. *J. Climate* **3**, 237–250 (1990).
- Angell, J. K. *Mon. Weath. Rev.* **114**, 1922–1930 (1986).
- Angell, J. K. & Korshover, J. *Mon. Weath. Rev.* **111**, 901–921 (1983).
- Karoly, D. J. *Geophys. Res. Lett.* **16**, 465–468 (1989).
- Kahl, J. D. et al. *J. geophys. Res.* (submitted).
- Timerev, A. A. & Egorov, S. A. *Meteorol. Gidrol.* No. 7, 50–56 (1991).

- Nagurnyi, A. P., Timerev, A. A. & Egorov, S. A. *Akad. Nauk SSSR, Dokl.* **319**, 1110–1113 (1991).
- Serreze, M. C., Kahl, J. D. & Schnell, R. C. *J. Climate*, **5**, 615–629 (1992).
- Serreze, M. C. et al. *J. geophys. Res.* **97**, 9411–9422 (1992).
- Walsh, J. E. *Mon. Weath. Rev.* **105**, 1527–1535 (1977).
- Kahl, J. D., Serreze, M. C., Shiotani, S., Skony, S. M. & Schnell, R. C. *Bull. Am. met. Soc.* **73**, 1824–1830 (1992).
- Serreze, M. C., Kahl, J. D. & Shiotani, S. *National Snow and Ice Data Center spec. Rep. No. 2* (CIRES, University of Colorado, 1992).
- Skony, S. M. thesis, Univ. of Wisconsin-Milwaukee (1992).
- Diaconis, P. & Efron, B. *Scient. Am.* **248**, 116–130 (1983).

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## Detection of crustal deformation from the Landers earthquake sequence using continuous geodetic measurements

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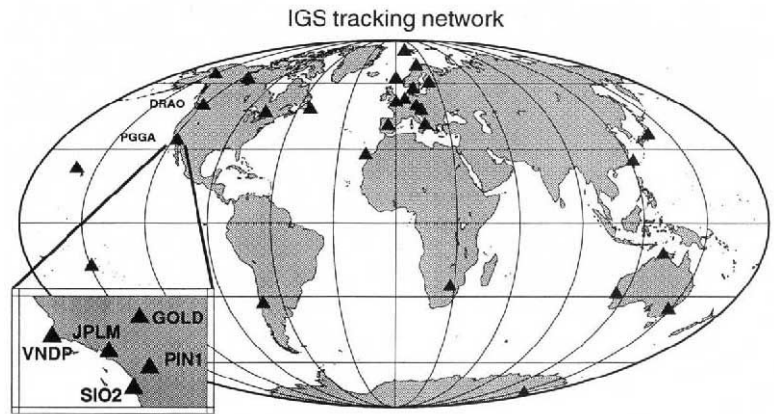
THE measurement of crustal motions in tectonically active regions is being performed increasingly by the satellite-based Global Positioning System (GPS)<sup>1,2</sup>, which offers considerable advantages over conventional geodetic techniques<sup>3,4</sup>. Continuously operating GPS arrays with ground-based receivers spaced tens of kilometres apart have been established in central Japan<sup>5,6</sup> and southern California to monitor the spatial and temporal details of crustal deformation. Here we report the first measurements for a major earthquake by a continuously operating GPS network, the Permanent GPS Geodetic Array (PGGA)<sup>7–9</sup> in southern California. The Landers (magnitude  $M_w$  of 7.3) and Big Bear ( $M_w$  6.2) earthquakes of 28 June 1992 were monitored by daily observations. Ten weeks of measurements, centred on the earthquake events, indicate significant coseismic motion at all PGGA sites, significant post-seismic motion at one site for two weeks after the earthquakes, and no significant preseismic motion. These measurements demonstrate the potential of GPS monitoring for precise detection of

precursory and aftershock seismic deformation in the near and far field.

The PGGA, established in southern California (Fig. 1) in the spring of 1990, is a network of five continuously operating GPS receivers providing an uninterrupted record of crustal motion in near real-time. At each site there is a precise P-code GPS receiver with its antenna mounted on a geodetic monument. Twenty-four hours of data are automatically collected from each site once a day; the operational analysis provides the site position averaged over the day (0–24 h Universal Time Coordinated, UTC), although finer resolution is possible. The precision of the daily relative position between any two sites in the network, based on long-term scatter of nearly 2 years of measurements, is ~5 mm in the horizontal and 10–20 mm in the vertical. To obtain this precision, and to achieve near-real-time solutions, we compute orbits for all GPS satellites, using data collected by a globally distributed network of about 25 permanent tracking stations<sup>10</sup> (Fig. 1), and corrections to tabulated predictions<sup>11</sup> of the orientation of the Earth's rotation axis (polar motion). The worldwide tracking network defines a global reference frame in which coordinates for the PGGA stations, in unstable southern California, can be computed with respect to rigid plate interiors (for example, the North American plate).

Here we report measurements of seismically induced displacements of the PGGA stations due to the Landers ( $M_w$  7.3, 11:58 UTC, 34.22° N, 116.43° W) and Big Bear ( $M_w$  6.2, 15:07 UTC, 34.21° N, 116.83° W) earthquakes of 28 June 1992. We examined the series of PGGA station positions in the 10-week period centred on the day of the earthquakes using a Kalman-filter formulation<sup>12</sup> to analyse the daily 24-hour PGGA solutions. For the day of the earthquakes, we computed the station positions separately from the 12 hours of data before the Landers earthquake and the 9 hours of data after the Big Bear event. The global tracking network was fairly extensive as the 3-month International GPS Service campaign<sup>13</sup> began on 21 June (Fig. 1). Displacements of the PGGA sites, listed in Table 1, were determined by examining the variation in the positions before and after the earthquakes with respect to a global reference frame defined by the coordinates of the tracking network stations. The horizontal displacements of the PGGA sites are

FIG. 1 The distribution of global GPS permanent tracking stations and PPGA sites used for our analysis of observations for the period May–August 1992. The closest tracking site outside California is DRAO in Penticton, British Columbia. All stations use Rogue SNR-8 GPS receivers except for two PPGA sites, SIO2 in La Jolla and VNDP at Vandenberg Air Force Base, which use Ashtech LX-II3 GPS receivers. The global tracking network is usually described by the acronyms CIGNET and FLINN, standing for Cooperative International GPS Network and Fiducial Laboratories for an International Natural science Network<sup>10</sup>, and more recently by the International GPS Service (IGS)<sup>13</sup>.



plotted in Fig. 2. The maximum horizontal displacement of 48 mm was detected at PIN1, located at the Piñon Flat Observatory (PFO) ~80 km from the seismic rupture zone. In Fig. 3 we take differences for the positions obtained with respect to the global reference frame and plot the daily record of relative horizontal positions between PIN1 and three other stations (Fig. 1). These were a global tracking site (DRAO) near Penticton, British Columbia, Canada, more than 1,700 km to the north and the global tracking station closest to the PPGA; the PPGA site (JPLM) in Pasadena; and the PPGA site (GOLD) at the NASA Deep Space Network Goldstone Complex.

An examination of the station displacements indicates that most of the deformation is due to the coseismic phase of the crustal deformation cycle<sup>14</sup>. The coseismic displacements appear clearly as step functions in the time series of daily station positions (Fig. 3). No significant pre-seismic signature is discernible from the five weeks of daily data taken before the earthquakes. There appears, however, to be a significant post-

seismic signature of  $0.9 \pm 0.3 \text{ mm day}^{-1}$  in our estimates of the relative positions of GOLD and PIN1 for 16 days after the earthquakes (Fig. 3). An examination of the displacements at the individual stations indicates that most of the post-seismic motion occurs at GOLD at a rate of  $0.7 \pm 0.3 \text{ mm day}^{-1}$ . (We were concerned that displacements observed at GOLD might stem from the construction of its antenna support, a 12-m-high microwave tower. This station, unlike the other PPGA stations which have very stable geodetic monuments, was neither installed nor operated primarily for monitoring tectonic motions, and might easily be recovering from the mainshock accelerations. Comparing the changing position of this monument with respect to another continuously operating GPS receiver 10 km away indicates, however, that the displacements were tectonic in origin.) The remainder of the motion, ~3 mm in total, occurs at PIN1, but the rate of displacement is not well determined from the data. Greater temporal detail and higher short-term resolution is provided by laser strainmeters at PFO; these show no pre-seismic signal, although post-seismic strains in the first 1–2 weeks following the earthquakes are consistent with our observations at PIN1. Further discrimination of the post-seismic signal will require the implementation of more refined analysis techniques. We intend to study the much longer time series of data before and after the earthquakes to look for possible pre-seismic signals and changes in the interseismic rate of deformation, and to understand better the error spectrum of continuous GPS data. We are currently investigating an increase in

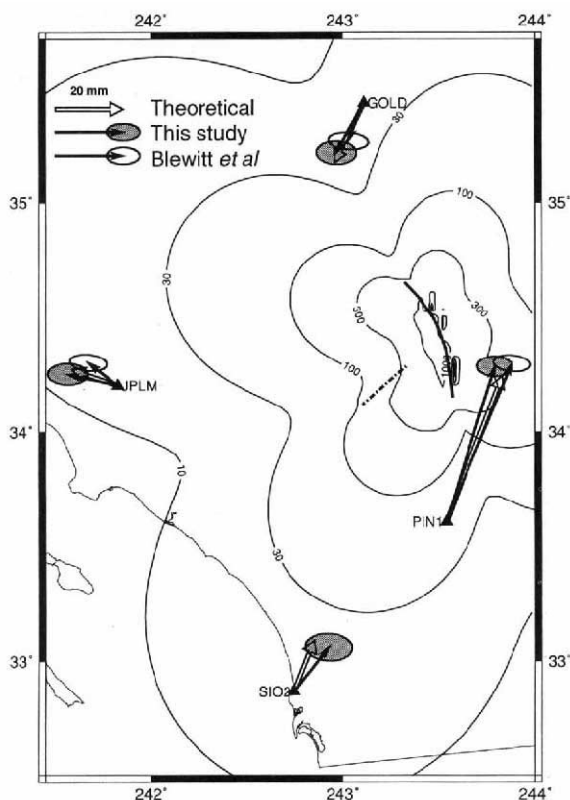


FIG. 2 Observed (solid arrows) and modelled (blank arrows) displacements at the PPGA stations, including 95% confidence ellipses. The observed displacements are calculated with respect to the reference frame defined by the positions and velocities of the global tracking stations, and include the total displacement estimated over the 5-week period after the earthquakes. Except for GOLD and a very small effect at PIN1, the displacements seem to be due entirely to coseismic motion. The total displacement observed at GOLD is 17 mm which includes ~6 mm of coseismic motion and 11 mm of apparent post-seismic motion over the 16 days following the earthquakes. For comparison, we show the displacements and 95% confidence ellipses (unshaded) computed by Blewitt *et al.*<sup>15</sup>. The contours of displacement magnitude, and the calculated displacements are for an elastic halfspace (all units are millimetres). The surface trace of the Landers rupture (heavy line) is composed of six segments with end points at latitude and longitude (degrees) (34.1500, 243.5708), (34.2250, 243.5625), (34.3625, 243.5375), (34.4500, 243.5000), (34.4958, 243.4750), (34.5917, 243.3917) and (34.6542, 243.3208). The magnitudes of slip are 1.5, 3.5, 1.0, 4.8, 5.2 and 0.8 m right-lateral respectively, with all segments from 0 to 15 km depth. The dashed line shows the surface trace assumed for the fault segment of the Big Bear earthquake, with 0.4 m of left-lateral slip on a segment from 3 to 15 km depth, and end points (34.1200, 243.1037) and (34.2884, 243.3297).



TABLE 1 Theoretical and observed displacements

| Site | Distance (km) | North (mm) |           | East (mm) |           | Amplitude (mm) |          |      | Azimuth (deg) |       |       | Vertical (mm) |          |
|------|---------------|------------|-----------|-----------|-----------|----------------|----------|------|---------------|-------|-------|---------------|----------|
|      |               | M          | O         | M         | O         | M              | O        | M/O  | M             | O     | M-O   | M             | O        |
| PIN1 | 89            | 42.8       | 45.6±1.2  | 16.2      | 14.0±2.1  | 45.8           | 47.7±2.4 | 0.96 | 20.7          | 17.1  | 3.6   | 13.8          | 9.6±6.4  |
| GOLD | 117           | -17.5      | -14.6±1.4 | -8.5      | -8.0±2.4  | 19.5           | 16.6±2.8 | 1.17 | 205.9         | 208.7 | -2.8  | 7.3           | 6.0±5.6  |
| JPLM | 155           | 4.7        | 3.4±1.3   | -13.4     | -14.7±2.4 | 14.2           | 15.1±2.7 | 0.94 | 289.3         | 283.0 | 6.3   | 0             | 0        |
| SIO2 | 185           | 15.1       | 13.0±1.7  | 6.0       | 10.1±2.8  | 16.2           | 16.5±3.3 | 0.98 | 21.7          | 37.8  | -16.1 | 8.9           | 10.3±8.2 |
| VNDP | 380           | 0.9        | 4.5±1.6   | -3.0      | -4.1±2.5  | 3.1            | 6.1±3.0  | 0.51 | 286.7         | 317.7 | -31.0 | 2.6           | 3.9±5.4  |

Total displacements in the horizontal components of the PGGA stations computed by a variable slip dislocation model (M) and estimated from 10 weeks of GPS observations (O) centred on the day of the earthquakes, and a comparison of the modelled and observed amplitudes and azimuths of the displacements. The displacements are computed with respect to the global reference frame defined by the global tracking stations (Fig. 1). The errors listed for the observed displacements are  $1\sigma$  values. The distance from the PGGA sites to the centroid of the model is given. For completeness we include the vertical displacements computed from the dislocation model and estimated from the GPS observations. In this case, the table gives the values for the motion of the PGGA sites relative to JPLM which has been shifted to 0 for both observation and model values. This requires adding 3.7 mm to the model value and 25.9 mm to the observed value. The vertical component errors are not well understood, however, so the agreement with the dislocation model may be fortuitous.

the data noise after the earthquakes (Fig. 3); this may partly be a meteorological effect related to the transition into summer, a phenomenon that has been observed in many years of positions determined by very-long-baseline interferometry in California.

An independent analysis of the PGGA and global tracking data by a group at the Jet Propulsion Laboratory (JPL) yielded

similar total displacements, as reported by Blewitt *et al.*<sup>15</sup>. The JPL group used the GIPSY GPS software<sup>16,17</sup> to process the data whereas we used the GAMIT/GLOBK GPS software<sup>12,18</sup>. The algorithms used in these two programs were independently developed and use different approaches. Furthermore, the two groups used different global reference frames and different subsets of the global tracking and PGGA data. We analysed ten weeks of data from all five PGGA sites, which included three Rogue SNR-8 GPS receivers and two Ashtech LX-II3 GPS receivers (at VNDP and SIO2), whereas Blewitt *et al.* analysed 8 weeks of Rogue receiver data only. A comparison of the results is shown in Fig. 2. The overlaps of our respective 95% confidence ellipses indicate good agreement at the three common sites.

In Fig. 2 we show the coseismic displacements computed from a variable slip dislocation model in an elastic halfspace<sup>19,20</sup>. The model for the Landers earthquake separates the 65-km rupture into six vertical planar segments (Fig. 2), oriented to coincide with the epicentre and the curvilinear pattern observed in the surface break and aftershock distribution<sup>21</sup>. The right-lateral slip on the southernmost three segments was calculated by averaging over each segment the observed surface offset and the slip distribution obtained by inverting strong-motion seismic data<sup>22</sup>. Slip on the northern three segments was obtained by fitting the displacement vector at GOLD. This required significantly less slip than observed along the surface break. The surface faulting observed geologically continued 10 km to the northwest of the main aftershock zone, suggesting that rupture along this segment of the fault is confined to shallow depths<sup>15,21</sup>. The Big Bear event was modelled as left-lateral rupture along a vertically dipping fault, oriented to coincide with the focal mechanism and aftershock distribution; the slip was chosen to agree with the seismic moment (H. Kanamori, personal communication).

There is good agreement between the theoretical and observed displacements at the PGGA sites (Table 1, Fig. 2). Our fit to the displacement vectors, although nonunique, suggests less slip in the northern half of the Landers rupture. In fact, the geodetic moment calculated using these far-field PGGA data is  $0.8 \times 10^{20}$  N m, which is generally less than moments obtained by independent means: near-field seismic ( $0.8\text{--}0.9 \times 10^{20}$  N m), geological ( $0.9 \times 10^{20}$  N m), near-field geodetic ( $1.0 \times 10^{20}$  N m) and teleseismic ( $1.1 \times 10^{20}$  N m)<sup>21,23</sup>. The comparatively low PGGA moment may be a manifestation of the influence of the mantle, which has higher values of elastic moduli than the crust<sup>24</sup>, so that far-field amplitudes of theoretically computed displacements are reduced relative to homogeneous halfspace models. In this case, the far-field is distances greater than the 30 km thickness of the crust.

These data show that continuous GPS arrays can provide reliable, precise and rapid determination of crustal motion, in particular seismically induced deformation. Although dense spatial coverage with such stations is not economically feasible

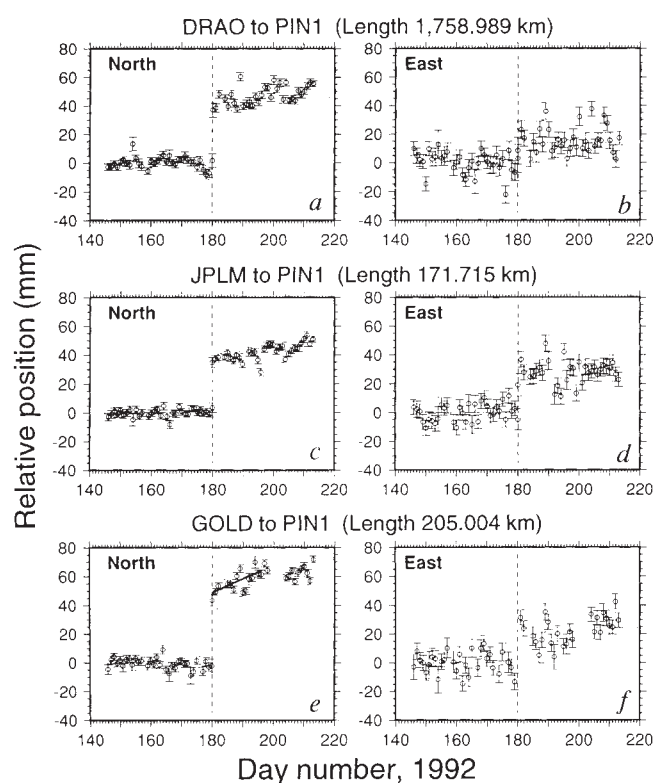


FIG. 3 Time series of daily horizontal relative positions (north and east components) over a 10-week period centred on the day of the earthquakes (day 180, 28 June 1992) for PIN1 relative to DRAO (a, b), JPLM (c, d) and GOLD (e, f). The dotted horizontal lines are determined from the weighted means of the data for the 5-week period before and after the earthquakes, and indicate the coseismic signature (these differ from the Kalman-filter estimates given in Table 1 by not more than 2.5 mm). The error bars have been scaled by a factor of two to account for the effects of correlated noise sources. These are not included in our statistical estimates of the uncertainties, which assume white-noise error sources. In e, we fit by least squares a straight line, with a slope of  $0.9 \pm 0.3$  mm day<sup>-1</sup>, to the apparent post-seismic signature for the 16 days following the earthquakes. The east component has a larger scatter than the north component because the integer-cycle phase ambiguities were not resolved to integer values<sup>16,18</sup> in the daily PGGA solutions.

at present, advances in GPS receiver technology will allow denser and more continuous measurements. □

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- Dixon, T. H. A. *Rev. Geophys.* **29**, 249–276 (1991).
- Hager, B. H., King, R. W. & Murray, M. H. *Rev. Earth planet. Sci.* **19**, 351–382 (1991).
- Sauber, J., Thatcher, W. & Solomon, S. C. *J. geophys. Res.* **91**, 12683–12693 (1986).
- Savage, J. *Geophys. Res. Lett.* **17**, 2113–2116 (1990).
- Shimada, S. *et al. Nature* **343**, 631–633 (1990).
- Shimada, S. & Bock, Y. *J. geophys. Res.* **97**, 12437–12455 (1992).
- Bock, Y. & Leppard, N. (eds) *Global Positioning System: An Overview* 40–56 (Springer, New York, 1990).
- Lindqwister, U., Blewitt, G., Zumbeke, J. & Webb, F. *Geophys. Res. Lett.* **18**, 1135–1138 (1991).
- Bock, Y. *GPS World* (Aster, Eugene, Oregon, 1991).
- Minster, J.-B., Hager, B. H., Prescott, W. H. & Schutz, R. E. *International Global Network of Fiducial Stations* (National Res. Council, National Academy Press, Washington DC, 1991).
- International Earth Rotation Service *Bulletins B 51–54* (Observatoire de Paris, 1992); *Bulletins A Vol. V* (U.S. Naval Observatory, 1992).
- Herring, T. H., Davis, J. L. & Shapiro, I. I. *J. geophys. Res.* **95**, 12561–12583 (1990).
- Beutler, G. *Eos* **73**, 134 (1992).
- Scholz, C. H. *The Mechanics of Earthquakes and Faulting* (Cambridge Univ. Press, 1990).
- Blewitt, G. *et al. Nature* **361**, 340–342 (1993).
- Blewitt, G. *J. geophys. Res.* **94**, 10187–10203 (1989).
- Blewitt, G. *Geophys. Res. Lett.* **17**, 199–202 (1990).
- Dong, D. & Bock, Y. *J. geophys. Res.* **94**, 3949–3966 (1989).
- Mansinha, L. & Smylie, D. E. *Bull. seism. Soc. Am.* **61**, 1433–1440 (1971).
- Okada, Y. *Bull. seism. Soc. Am.* **75**, 1135–1154 (1985).
- Landers Earthquake Response Team *Science* (submitted).
- Kanamori, H., Thio, H.-K., Dreger, D., Hauksson, E. & Heaton, T. *Geophys. Res. Lett.* **19**, 2267–2270 (1992).
- Hudnut, K. W. *et al. Eos* **73**, 365 (1992).
- Rybicki, K. *Bull. Seism. Soc. Am.* **61**, 79 (1971).

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## Absolute far-field displacements from the 28 June 1992 Landers earthquake sequence

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ON 28 June 1992, the largest earthquake in California in 40 years (surface-wave magnitude  $M_s = 7.5$ ) occurred near the small town of Landers, in southeastern California, and was followed three hours later by the nearby  $M_s$  6.5 Big Bear earthquake<sup>1</sup>. Fortuitously, the Landers earthquake sequence coincided with the first week of the official three-month test period of the International Global Positioning System and Geodynamics Service<sup>2</sup> (IGS), giving us an unprecedented opportunity to detect absolute pre-, co- and post-seismic displacements at a distance of 50–200 km from the main rupture with millimetre-level precision. Mutual and independent confirmation of some of our geodetic results are demonstrated by Bock *et al.* in this issue<sup>3</sup>. For the Landers earthquake, the observed displacements indicate that the depth of the bottom of the rupture is shallower towards the northern end, displacements were dominantly symmetric, and the rupture extended further south on the Johnson Valley fault than has been mapped on the basis of surface ground offsets. The combined geodetic moment for the Landers and Big Bear earthquakes ( $1.1 \times 10^{20}$  N m<sup>-1</sup>) agrees well with teleseismic estimates.

The Landers and Big Bear earthquakes and their aftershocks occurred along faults that form a triangle bounded to the south-west by the San Andreas fault (Fig. 1). Extensive surface rupture resulting from these events has been reported along the Johnson

Valley and Camp Rock/Emerson faults. Ground breakage occurs along a 70-km stretch of these faults, reaching a maximum surface offset of 6.7 m (ref. 1). The Landers earthquake occurred toward the southern end of the hypothesized 'Mojave shear zone', which trends N35°W across the Mojave Desert, into Owens Valley and the northern Basin and Range province<sup>4,5</sup>. This zone reportedly carries 7–8 mm yr<sup>-1</sup> of the relative motion between the Pacific and North American plates, and may be a manifestation of a subcrustal fault<sup>4,5</sup>. Aftershocks following the Landers earthquake line up along this apparent shear zone from as far south as the San Andreas fault, to further north than our station GOLD shown in Fig. 1. The pattern of aftershocks is sparse on the Camp Rock fault segment which underlies the northernmost 13 km of the visible surface rupture<sup>6</sup>. Using a new geodetic tool for earthquake studies, we have estimated permanent surface displacements in southern California due to the cumulative effect of events on 28 June, and show that geodetic methods provide valuable information on aspects of the rupture mechanism not available with other techniques. We also offer an explanation for the unexpected lack of aftershocks on the Camp Rock fault.

Since 21 June 1992, a worldwide network of stations has been routinely receiving precise microwave ranging data from the United States Department of Defence's 18-satellite Global Positioning System (GPS)<sup>7</sup>, and transmitting the data to IGS data centres to be made available to analysis centres and geodynamics researchers. Regional GPS networks benefit from the precise orbit determination and reference frame stability supplied by an extensive tracking network<sup>8–10</sup>. A regional array of receivers operated jointly by the Jet Propulsion Laboratory (JPL) and Scripps Institution of Oceanography has been operational in southern California since 1990 (ref. 11). A simultaneous analysis of GPS data from the California array combined with the global network data has allowed us to estimate the absolute displacements, in the international terrestrial reference frame<sup>1,2</sup> (ITRF), of three stations located within 50–200 km from the Landers earthquake rupture, with 2-mm precision in the horizontal plane.

To reduce systematic errors that can be introduced by mixing different types of GPS receivers and antennas, we have analysed

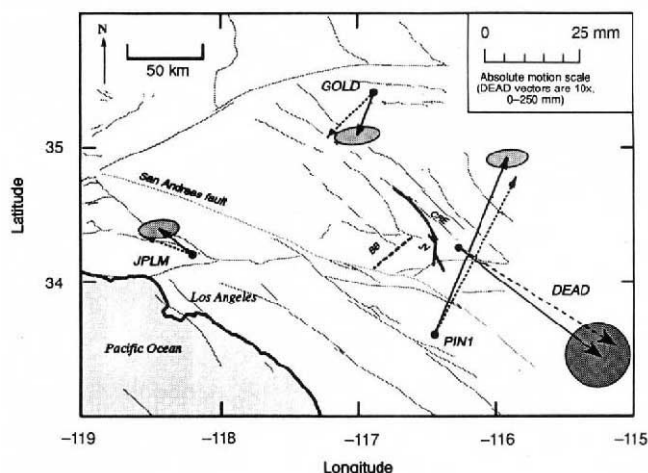


FIG. 1 Map showing absolute motions of Goldstone (GOLD), Pasadena (JPLM), Pinyon Flat (PIN1) and Deadman (DEAD). Solid arrows are the observed displacements with 95% confidence regions. The vectors and confidence region for DEAD is shown at 0.1 times the scale of the other stations. The model displacements, assuming an elastic half-space, are shown as dashed arrows. The surface trace of the model of the Landers earthquake is shown by the solid heavy line. Dashed heavy line (BB) is the trace of the fault used to model the Big Bear earthquake. Shaded solid and dashed lines are active faults in the region: JV is the Johnson Valley fault; CRE is the Camp Rock/Emerson fault.



data from the 28-station homogeneous subset of the global network that uses 'Rogue' receivers (only three of which were operating in California at the time of the earthquake). Since early May 1992, we have been routinely producing high-precision estimates of GPS satellite orbits, station coordinates<sup>13</sup> and the Earth's pole of rotation<sup>14</sup>.

Provided that the wobble of the Earth's pole is monitored and that we model the known tidal deformations of the solid Earth, the receiver coordinates of the global network can be defined such that distances between all stations and distances between each station and the Earth's centre of mass are consistent with the GPS observations. 'Absolute displacements' are defined here as the permanent change in these station coordinates due to the combined effect of the Landers and Big Bear earthquakes. This concept is emphasized because previous geodetic techniques have only been sensitive to relative motion between stations (which is inherently ambiguous). This ability to measure 'absolute displacements' allows us to investigate symmetric versus asymmetric displacements. In most models of earthquake ruptures so far (including our own) each side of the fault is assumed to move half the total slip distance. Asymmetric displacements could have profound implications for the evaluation of seismic hazard of areas affected by the fault<sup>15</sup>.

The analysis presented here is based on data retrieved from 1 June to 26 July 1992 (that is,  $\pm 4$  weeks from 28 June). A subset of the global stations were held fixed to their ITRF coordinates<sup>10</sup>, which have been shown to be consistent with GPS observations<sup>13</sup>. The orientation of the Earth's pole of rotation was estimated every 24 hour interval. Two types of solution are used here. (1) To indicate the precision of our solutions, and to test for pre- and post-seismic motion, station coordinates were estimated independently every day. Relative positions are shown in Fig. 2. We include in these examples the estimates of JPLM with respect to DRAO at Penticton, British Columbia, Canada, which is the closest station (at 1,700 km) safely outside the zone of deformation. The several-millimetre northern displacement of JPLM relative to DRAO is evident. We conclude from this figure that we did not detect significant pre- or post-seismic motion of the three permanent stations at the few millimetre level. (2) To estimate rigorously co-seismic displacement, we reduced the data in a single solution, for which we assumed that all stations outside of California were stationary during this 8-week period, and the positions of the California stations were modelled as a step-function centred on 28 June. For simplicity, data from 28 June were not used. Unmodelled tectonic plate motion over this period would result in insignificant systematic errors in the step-function estimates at the 1 mm level. The formal standard deviations of the estimates were scaled by a factor of 2 to make them more consistent with the observed r.m.s. scatter of daily solutions, which was typically 3 mm for the north components, 5 mm for the east components, and 10–15 mm for the vertical components. Table 1 contains estimates of the absolute displacements for the three continuously operating California sites analysed.

In addition to the displacements at the three permanently operating receivers, displacements were estimated for Deadman Lake, a very-long-baseline interferometer (VLBI) station, which we occupied using GPS within hours of the main shock. These

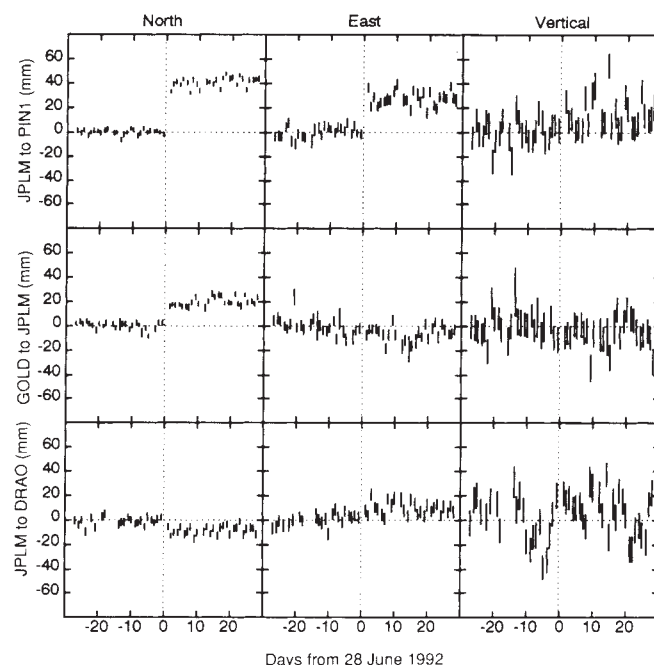


FIG. 2 Daily estimated coordinates of Goldstone (GOLD) and Pinyon Flat (PIN1) relative to Pasadena (JPLM) shown in local components (north, east and vertical), with their one-standard deviation error bars. The lowest three plots show the displacement of JPLM relative to Penticton (DRAO), British Columbia, over 1,700 km away. Despite the long distance, the step function in the north component is clearly evident (in this plot, DRAO appears to move south relative to JPLM). These plots are for illustration only; the actual displacement estimates in Table 1 are based on a step-function model for stations in California, and are absolutely determined with respect to a consistently defined Earth-fixed, corotating reference frame.

displacements are based on the difference between GPS-estimated coordinates and the VLBI coordinates for this station (both sets of coordinates being consistently expressed in the ITRF). Figure 1 indicates the estimated horizontal displacements for the 4 California stations. To within their 95% confidence ellipses, the displacement estimates for JPLM, PIN1 and GOLD have been mutually and independently confirmed by Bock *et al.*<sup>3</sup>.

Forward modelling using a homogeneous elastic half-space and perturbations to a fault model derived from TERRAScope inversion<sup>16</sup> and measured surface offsets<sup>6</sup> was used to calculate displacements of the four stations JPLM, PIN1, GOLD and DEAD. The model includes eight fault planes for the Landers quake and one fault plane for the Big Bear quake. Details of the fault plane locations and slips are given in Table 2. To allow the modelled displacements to come close to the observed displacements, the moment release from the northern end of the Landers quake must be small. In view of the measured surface offsets in this region, the small moment in turn requires the depth of rupture to be shallow, as seismic moment is

TABLE 1 Absolute displacements of California GPS stations

| Station            | Approx. coordinates |           | Estimates (mm) |           |          | Model (mm) |       |          |
|--------------------|---------------------|-----------|----------------|-----------|----------|------------|-------|----------|
|                    | Latitude            | Longitude | East           | North     | Vertical | East       | North | Vertical |
| Pasadena (JPLM)    | 34° 12'             | 241° 50'  | -9 ± 2         | 7 ± 1     | -8 ± 5   | -13        | 6     | -4       |
| Goldstone (GOLD)   | 35° 26'             | 243° 07'  | -5 ± 2         | -11 ± 1   | -4 ± 5   | -13        | -10   | 2        |
| Pinyon Flat (PIN1) | 33° 37'             | 243° 32'  | 19 ± 2         | 46 ± 1    | 8 ± 5    | 24         | 39    | 10       |
| Deadman (DEAD)     | 34° 15'             | 243° 43'  | 384 ± 35       | -302 ± 34 | 100 ± 31 | 397        | -280  | 27       |

TABLE 2 Details of fault-plane assumptions for elastic half-space model

| Faults* | Dip<br>(deg) | Rake<br>(deg) | Width<br>(km) | Length<br>(km) | Depth<br>(km) | Slip<br>(m) | Starting<br>longitude<br>(deg) | Starting<br>latitude<br>(deg) | Ending<br>longitude<br>(deg) | Ending<br>latitude<br>(deg) |
|---------|--------------|---------------|---------------|----------------|---------------|-------------|--------------------------------|-------------------------------|------------------------------|-----------------------------|
| EP      | 90.0         | 180.0         | 15.0          | 15.24          | 0.0           | 0.5         | -116.376                       | 34.083                        | -116.439                     | 34.210                      |
| JVs     | 90.0         | 180.0         | 12.0          | 18.63          | 0.0           | 4.2         | -116.447                       | 34.124                        | -116.432                     | 34.292                      |
| JVn     | 90.0         | 180.0         | 15.0          | 7.93           | 0.0           | 3.5         | -116.434                       | 34.291                        | -116.485                     | 34.349                      |
| Kik     | 90.0         | 180.0         | 15.0          | 5.38           | 0.0           | 2.5         | -116.455                       | 34.310                        | -116.447                     | 34.358                      |
| HVs     | 90.0         | 180.0         | 8.0           | 10.30          | 0.0           | 3.5         | -116.425                       | 34.315                        | -116.470                     | 34.400                      |
| HVn     | 90.0         | 180.0         | 8.0           | 15.72          | 0.0           | 3.3         | -116.470                       | 34.400                        | -116.549                     | 34.526                      |
| E       | 90.0         | 180.0         | 5.0           | 12.75          | 0.0           | 5.5         | -116.551                       | 34.524                        | -116.633                     | 34.617                      |
| CR      | 90.0         | 180.0         | 3.0           | 13.03          | 0.0           | 1.0         | -116.634                       | 34.616                        | -116.733                     | 34.701                      |
| BB      | 90.0         | 0.0           | 15.0          | 35.07          | 2.0           | 0.8         | -116.632                       | 34.316                        | -116.901                     | 34.093                      |

\* EP: Eureka Peak; JVs: Johnson Valley (south); JVn: Johnson Valley (north); Kik: Kikapoo; HVs: Homestead Valley (south); HVn: Homestead Valley (north); E: Emerson; CR: Camp Rock; BB: Big Bear

proportional to the product of slip and rupture area. In our model the Homestead Valley (north), Emerson and Camp Rock faults have a maximum depth of faulting of 8.0, 5.0 and 3.0 km respectively. To fit the observed displacements of DEAD, we have modelled the southern end of the Johnson Valley (south) fault to be ~8 km south of the recognized surface rupture. This extension may more properly coincide with the Burnt Mountain fault<sup>6,17</sup>.

For the Landers earthquake only, teleseismic data yield a moment of  $1.1 \times 10^{20} \text{ N m}^{-1}$  (ref. 6); inversions of TERRAScope data yield  $0.8 \times 10^{20} \text{ N m}^{-1}$  (ref. 6); geological observations yield  $0.9 \times 10^{20} \text{ N m}^{-1}$  (ref. 6); and our model has a moment of  $0.9 \times 10^{20} \text{ N m}^{-1}$ .

The differences between our model and the observed displacements may be attributed to a combination of inaccuracies in our fault model and possibly inhomogeneous response of the crust. Our GPS results strongly suggest that rupture under the northernmost segment of the surface break is surprisingly shallow (extending ~3 km below the surface). This may explain the paucity of aftershocks on the Camp Rock fault. The displacements derived from our (symmetric rupture) model fall outside the 95% confidence ellipses at PIN1 and GOLD by 6% and 15% of the observed total displacement at the respective site. These discrepancies may be explained by some combination of asymmetric rupture, inhomogeneous material properties, and mis-modelling of fault parameters. □

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- Hauksson, E. L. et al. *Cal. Inst. Technol. Preliminary UPDATE Rep.* (1992).
- Mueller, I. I. *Proc. 2nd Int. Symp. Precise Positioning with GPS, XIVIII-LXVI* (Canadian Institute of Surveying and Mapping, Ottawa, 1990).
- Bock, Y. et al. *Nature* **361**, 337-340 (1993).
- Savage, J. C., Lisowski, M. & Prescott, W. F. *Geophys. Res. Lett.* **17**, 2113-2116 (1990).
- Sauber, J., Thatcher, W., & Solomon, S. *J. geophys. Res.*, **19**, 12683-12693, (1986).
- Sieh et al. *Science* (in the press).
- Dixon, T. H. *Rev. Geophys.* **29**, 249-276 (1991).
- Schutz, B. E. et al. *Geophys. Res. Lett.* **17**, 643-646 (1990).
- Larson, K. M., Webb, F. H. & Agnew, D. C. *J. geophys. Res.* **96**, 16567-16584 (1991).
- Frey Mueller, J. T. & Kellogg, J. N. *Geophys. Res. Lett.* **17**, 207-210 (1990).
- Lindqvister, U. J. et al. *Geophys. Res. Lett.* **18**, 1135-1138 (1991).
- IERS A. Rep. 1991 (IERS Central Bureau, Observatoire de Paris, 1992).
- Blewitt, G. et al. *Geophys. Res. Lett.*, **19**, 853-856 (1992).
- Lindqvister, U. J., Freedman, A. P. & Blewitt, G. *Geophys. Res. Lett.* **19**, 845-848 (1992).
- Horowitz, F. & Stewart, C. *Eos* **73**, No. 43, 373-374 (1992).
- Kanamori, H., Thio, H., Dreger, D., Hauksson, E. & Heaton, T. *Geophys. Res. Lett.* **19**, 2267-2270 (1992).
- Trieman, J. *Southern California Sect. Eng. Geologists, Annual Field Trip Guidebook*, 19-22 (1992).

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## Do virtual geomagnetic poles follow preferred paths during geomagnetic reversals?

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VIRTUAL geomagnetic poles (VGPs) recorded in sediments during reversals of the Earth's magnetic field show an apparent preference for two antipodal sectors of longitude<sup>1-3</sup>, not only in records of the same reversal from different sites, but also in records of different reversals. If preferred bands really have persisted from one reversal to the next, this would imply that the mantle exerts a significant control over the reversal process<sup>4</sup>. Here we analyse the available database of reversal records from the past 12 Myr, using a statistical test specifically designed to test the hypothesis of two preferred antipodal longitudinal bands. Our analysis shows that the records, taken as a group of independent observations, do show an overall preference for two antipodal longitudinal bands. However, the site longitudes are also strongly grouped<sup>5</sup>, and a comparison of the transitional VGP longitudes with site longitudes shows an unlikely grouping under the hypothesis of a genuine geographical preference for transitional VGPs. We conclude that it is premature to accept the hypothesis of mantle control over the core during geomagnetic reversals.

Laj et al.<sup>4</sup> presented a visually compelling diagram showing all of the individual VGP positions, and linked the preferred longitudinal sectors to lateral inhomogeneities in the lower mantle. However, the hypothesis of preferred sectors was not based on an objective analysis of the data: it relied strongly on a visual perception which may have been biased by the presentation.

Valet et al.<sup>5</sup> have recently disputed the interpretation of preferred longitude sectors for the transitional VGPs, basing their conclusions on a  $\chi^2$  analysis of a parameter they refer to as the MVL (mean value of longitude) for each record. Given  $n$  VGP unit vectors  $\mathbf{v}_i = (x_i, y_i, z_i)^T$  in a transitional record the MVL for that record is simply a weighted mean of the  $i$  longitudes, the weight for each  $\mathbf{v}_i$  being the cosine of the latitude of  $\mathbf{v}_i$ . Thus

$$\text{MVL} = \arctan \left( \frac{\sum_{i=1}^n y_i / \sum_{i=1}^n x_i}{\sum_{i=1}^n z_i / \sum_{i=1}^n x_i} \right)$$

Clearly it is necessary to use some form of central measure to categorize each record with a single parameter for analysis, and the MVL seems the most natural choice. Laj et al.<sup>6</sup>, in a more



recent database, have chosen to use the (interpolated) point EC (Equator crossing) where the transitional VGP path crosses the Equator. The EC uses less information from the record than does the MVL, and the two often differ by up to 20° for the same record. However, it should matter little which is used, provided one is consistent.

The  $\chi^2$  test used by Valet *et al.*<sup>5</sup> is unreliable with small samples (as is the situation here) and in any case is not a powerful test for the type of hypothesis suggested by Laj *et al.*<sup>4</sup>. Consequently the whole question of preferred bands for transitional VGPs demands a better analysis, based on a tool that is more powerful for the Laj *et al.*<sup>4</sup> hypothesis and, at the same time, less susceptible to problems with a small sample size. Laj *et al.*<sup>7</sup> have now used the Rayleigh, Rao and Watson tests (see, for example, ref. 8), which are much better than a  $\chi^2$  test, on their most recent database of EC values. However, none of these tests has an hypothesis of preferred sectors as an alternative to the null hypothesis of uniform randomness. Thus the specific hypothesis suggested by Laj *et al.*<sup>4</sup> has yet to be used in a test of the hypothesis. We have designed a test that does specifically use this hypothesis as the alternative, and present the results here.

The model in Fig. 1 indicates how the analysis operates to mimic the visual process on which the Laj *et al.*<sup>4</sup> hypothesis was based, and then to perform a statistical test to determine the significance of the observation. The MVL or EC values are placed around the circumference and the rotator turned until it covers the maximum number  $N_{\text{obs}}$  of observed values.  $N_{\text{obs}}$  is then the test statistic, for which we require a distribution under the null hypothesis that the distribution of MVLs or ECs is uniform random around the Equator. This is easily achieved with simulation by repeating the process many times (10,000 repetitions were used for each test in this study), each time on a different sample of values drawn at random from a uniform distribution around the Equator to give a value  $N_{\text{sim}}$ . Obviously one may tune the tool to the specific problem by choosing either one or two lobes on the rotator. The angle  $\theta$  of the rotator lobes is a parameter to be specified as part of the alternative hypothesis. Rothman<sup>9</sup> provides an exact solution for this test for certain sector sizes with the number of observations in the sector exceeding half the total.

The database used was the most recent compilation of Laj *et al.*<sup>6</sup>, with a total of 50 equatorial crossings. The individual transitions are represented by different numbers of observations (from 1 to 10), so it is probably incorrect to treat the individual records as independent observations with equal weight. However, in the absence of more data, there seems to be little option but to use equal weighting. To simplify the choice of  $\theta$ , the angle of the rotator lobes, we decided to restrict the choice to sectors of size  $(360^\circ/L)$  with  $L$  an integer. The smallest such sector that 'covers' the apparent concentration of paths is 60°, so this value was used for  $\theta$ . Although most of the concentration is in the one lobe, the statistical test shows that the grouping of

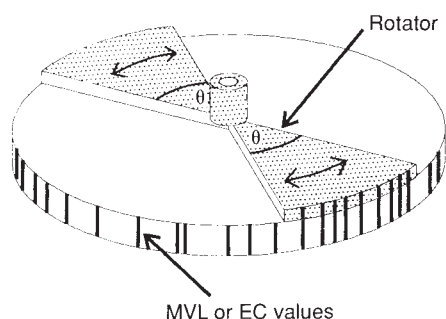


FIG. 1 Model for the statistical tool.

TABLE 1 Summary of statistical tests

| Data            | Preferred band boundaries                                              | Centre of preferred band   | $N_{\text{obs}}$ | Probability $N_{\text{sim}} \geq N_{\text{obs}}$ |
|-----------------|------------------------------------------------------------------------|----------------------------|------------------|--------------------------------------------------|
| EC values       | $-94^\circ \rightarrow -35^\circ$<br>$86^\circ \rightarrow 145^\circ$  | $-64^\circ$ or $116^\circ$ | 32 out of 50     | 0.04%                                            |
| Site longitudes | $-26^\circ \rightarrow 33^\circ$<br>$-147^\circ \rightarrow 154^\circ$ | $4^\circ$ or $-176^\circ$  | 35 out of 50     | 0.00%                                            |
| EC-site         | $-114^\circ \rightarrow -55^\circ$<br>$66^\circ \rightarrow 125^\circ$ | $-84^\circ$ or $96^\circ$  | 28 out of 50     | 2.62%                                            |

Probabilities are shown to 0.01% simply to indicate that 10,000 simulations were used.

observations is less likely for two antipodal lobes than for a single lobe, so a double-lobe test has been used throughout.

A summary of the statistics from the double-lobe tests is given in Table 1. It is very unlikely that so many ECs would have fallen into the preferred bands had the underlying distribution been uniform random. We infer that the conclusion of Valet *et al.*<sup>5</sup> is not supported, but that there is a preferential grouping of ECs in two antipodal longitudinal bands (one being over the Americas) of width about 60°.

Before accepting the hypothesis of mantle control over the core it is important to test whether this preferential grouping of ECs could derive from some other cause, such as a grouping of site longitudes together with a nonuniformity in the distribution of the angle between site longitude and EC. Using the same alternative hypothesis (two antipodal lobes with  $\theta = 60^\circ$ ), we have applied the test to the site longitude data. The summary in Table 1 shows that the site longitudes are even more strongly grouped than the EC values. This is a cause for concern. Naturally these two groupings imply that there must also be a preferential grouping of the angle between EC and site longitude (EC-site) as noted by Valet *et al.*<sup>5</sup>, which is confirmed by the summary statistics in Table 1. The fact that the centres of the preferred bands for (EC-site) are almost exactly at  $\pm 90^\circ$  is also of concern, because this could easily be caused by (for example) the nonlinearity of the VGP transformation<sup>10</sup>.

The analysis so far suggests two hypotheses: hypothesis 'Exciting' and hypothesis 'Dull'. Under hypothesis Exciting, there is a genuine geographically preferred band for ECs (that is, the distribution of ECs is independent of site longitude), and the grouping of (EC-site) is simply caused by the unfortunate grouping of site longitudes. Under hypothesis Dull, there is some mechanism that causes a bias of (EC-site) towards  $\pm 90^\circ$  (that is, the distribution of ECs is dependent on site longitude) so that the strong grouping of site longitudes produces an apparent, but artificial, geographical grouping of ECs. To see which of these hypotheses the data favour, the data have been split into two main groups, each with two subgroups. Group (a) contains those records from sites that fall within the two 60° preferred-site bands and group (b) contains the remainder. Within each of these groups, subgroup 1 is those records for which EC is in the preferred-EC bands and subgroup 2 is the remainder.

In group (a), 26 out of 35 fall into subgroup 1. Under hypothesis Exciting, the probability of a record falling into subgroup 1 is the same for both groups, so we would expect that about 11 of the 15 records in group (b) would fall into subgroup 1. However, almost the reverse happens: 9 of the 15 records in group (b) fall into subgroup 2, which is entirely consistent with hypothesis Dull.

Given the small number of records, is this observation significant? A  $2 \times 2$  contingency table may be used to see if the two sets of observations (26, 9) and (6, 9) could have been drawn from the same population. The test statistic has a value of 5.36 which, under the null hypothesis, has a  $\chi^2$  distribution on one

degree of freedom. This gives a probability of only 0.02, so even though the sample size is small, it is unlikely that we would obtain the present observations under hypothesis Exciting. A similar conclusion is reached if we use the MVL database of Valet *et al.*<sup>5</sup>.

Recent volcanic data and analyses<sup>11,12</sup>, although apparently supportive of hypothesis Exciting, are as yet not compelling. This is because the database suffers from problems similar to the sedimentary database, so careful analysis is required to exclude causes such as the correlations now apparent in the sedimentary database.

The most important issue to resolve at this stage is the cause of the apparent  $\pm 90^\circ$  preferential grouping for (EC-site). Two points should be noted. First, because the preferred-EC bands are  $60^\circ$  wide, only a small bias away from uniform randomness towards  $\pm 90^\circ$  in (EC-site) is needed. For example, one can

construct systematic rock magnetic effects such as increased inclination errors in sediments at low field intensity<sup>13</sup> that would contribute to such a bias. Furthermore, Egbert<sup>10</sup> has shown that, under a wide range of circumstances, one could expect VGP longitude distributions to peak  $\pm 90^\circ$  in longitude away from the observation site. Such possibilities must be seriously considered. Second, until there are sufficient data to perform testing for each reversal independently, and then test composite results from the different reversals, any conclusion must be considered tentative. Notwithstanding this second point, it seems premature to embrace hypothesis Exciting when the very data on which this hypothesis is based do not support it. More data are certainly required. Further data that fall into group (1a) are almost counterproductive, however, and it is crucial that further data be obtained from sites that fall well outside the present preferred-site bands.  $\square$

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1. Tric, E. *et al.* *Phys. Earth planet. int.* **65**, 319–336 (1991).
2. Gubbins, D. *Eos* **72**, 132 (1991).
3. Clement, B. M. & Kent, D. V. *Geophys. Res. Lett.* **18**, 81–84 (1991).
4. Laj, C. *et al.* *Nature* **351**, 447 (1991).
5. Valet, J.-P., Tucholka, P., Courtillot, V. & Meynadier, L. *Nature* **356**, 400–407 (1992).
6. Laj, C. *et al.* *Geophys. Res. Lett.* **19**, 2003–2006 (1992).
7. Laj, C. *et al.* *Nature* **359**, 111–112 (1992).
8. Mardia, K. V., *Statistics of Directional Data* (Academic, London, 1972).

9. Rothman, E. D. *Sankhya* **A34**, 23–32 (1972).
10. Egbert, G. *Geophys. Res. Lett.* **19**, 2353–2356 (1992).
11. Hoffman, K. A. *Nature* **359**, 789–794 (1992).
12. Constable, C. *Nature* **358**, 230–233 (1992).
13. Barton, C. E. *et al.* *Geophys. J. R. astr. Soc.* **61**, 355–377 (1980).

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## Expansion of C4 ecosystems as an indicator of global ecological change in the late Miocene

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THE most common and the most primitive pathway of the three different photosynthetic pathways used by plants is the C3 pathway, or Calvin cycle, which is characterized by an initial CO<sub>2</sub> carboxylation to form phosphoglyceric acid, a 3-carbon acid. The carbon isotope composition ( $\delta^{13}\text{C}$ ) of C3 plants varies from about  $-23$  to  $-35\%$ <sup>1–3</sup> and averages about  $-26\%$ . Virtually all trees, most shrubs, herbs and forbs, and cool-season grasses and sedges use the C3 pathway. In the C4 pathway (Hatch–Slack cycle), CO<sub>2</sub> initially combines with phosphoenol pyruvate to form the 4-carbon acids malate or aspartic acid, which are translocated to bundle sheath cells where CO<sub>2</sub> is released and used in Calvin cycle reactions<sup>1–4</sup>. The carbon isotope composition of C4 plants ranges from about  $-10$  to  $-14\%$ , averaging about  $-13\%$  for modern plants<sup>1–3</sup>. Warm-season grasses and sedges are the most abundant C4 plants, although C4 photosynthesis is found in about twenty families<sup>5</sup>. The third photosynthetic pathway, CAM, combines features of both C3 and C4 pathways. CAM plants, which include many succulents, have intermediate carbon isotope compositions and are also adapted to conditions of water and CO<sub>2</sub> stress. The modern global ecosystem has a significant component of C4 plants, primarily in tropical savannas, temperate grasslands and semi-desert scrublands. Studies of palaeovegetation from palaeosols and palaeodiet from fossil tooth enamel indicate a rapid expansion of C4 biomass in both the Old World and the New World starting 7 to 5 million years ago. We propose that the global expansion of C4 biomass may be related to lower atmospheric carbon dioxide levels because C4 photosynthesis is favoured over C3 photosynthesis when there are low concentrations of carbon dioxide in the atmosphere.

Fossil soils and fossil tooth enamel are important indicators of the presence of C4 biomass in local ecosystems. The carbon

isotope composition of soil carbonate is related to the isotope composition of the local biomass, being enriched in  $^{13}\text{C}$  by about 14 to 17% compared to the local biomass<sup>6,7</sup>; the carbon isotope composition of tooth enamel is enriched in  $^{13}\text{C}$  by about 12% compared to the diet<sup>8,9</sup>. Palaeosol (fossil soil) carbonate and fossil tooth enamel are robust indicators of palaeoenvironment<sup>10–13</sup> and palaeodiet<sup>14–16</sup>, respectively, and have been shown to retain their isotope composition through diagenesis<sup>17,18</sup>. The large range in the  $\delta^{13}\text{C}$  values for C3 plants (where  $\delta^{13}\text{C} = [(^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{PDB}} - 1] \times 1,000$  and PDB is the stable isotope reference standard) results in some ambiguity in interpretation of the  $\delta^{13}\text{C}$  values for pedogenic carbonate and tooth enamel. Under conditions of moisture stress, the isotope composition of C3 plants can be several % enriched in  $^{13}\text{C}$  (refs 3, 19, 20), so that ecosystems with  $\delta^{13}\text{C}$  values as high as  $-23\%$  could be essentially pure C3 ecosystems, with resultant  $\delta^{13}\text{C}$  of soil carbonate of  $-8\%$  and tooth enamel of  $-10\%$ . As many animals are highly selective in their dietary intake,  $\delta^{13}\text{C}$  values as high as  $-8$  or  $-9\%$  could result from a diet of C3 plants in semi-arid regions that are isotopically enriched in  $^{13}\text{C}$  compared to more mesic ecosystems.  $\delta^{13}\text{C}$  values of  $-20\%$  have been reported for C3 plants growing in arid environments where stomatal conductance is limited<sup>21</sup>.

Palaeosols from Pakistan show a very well defined change in the isotope composition of palaeosol carbonate from about  $-9$  to  $-12\%$  beginning at about 7.4 Myr, to  $-2$  to  $+2\%$  by about 5 Myr<sup>12</sup>. (Figure 1 shows data from ref. 12 as a five-point running average, with a sampling density of about 1 palaeosol per 130,000 years over 17 Myr.) The palaeosol data from Pakistan indicate a virtually pure C3 ecosystem before 7 Myr, which was replaced by an ecosystem dominated by C4 biomass by 5 Myr. It is unlikely that more than a small fraction of the biomass could have been using the C4 or CAM photosynthetic pathways before 7 Myr. Because of the possibility of C3 plants being isotopically enriched in semi-arid environments, which are those in which pedogenic carbonate formation is favoured<sup>22,23</sup>, even the most positive  $\delta^{13}\text{C}$  values for Pakistan palaeosols before 7.5 Myr ago represent essentially pure C3 ecosystems.

The isotope composition of fossil tooth enamel from mammals from the Siwaliks in Pakistan and from horses in western North America indicate that C4 plants were an important part of the diet starting between 7 and 6 Myr ago (Fig. 1), which is the same time that C4 biomass became important in Pakistan. Major



faunal turnover, including the local extinction of hominoids, occurred in the Siwaliks between 7 and 5 Myr ago<sup>24-27</sup>. The shift in the  $\delta^{13}\text{C}$  of enamel from North American horse teeth does not correspond to the noted change in hypsodonty that occurred in the early to middle Miocene<sup>28</sup>. This observation will require a revision in the interpretation of mammalian dentition and its relationship to the spread of savanna ecosystems in the Miocene. Preliminary studies of palaeosol carbonate from East Africa suggest that C4 biomass was of minor importance in the Miocene, but expanded significantly in the Pliocene and Pleistocene<sup>13</sup>.

The palaeosol carbonate and fossil tooth enamel data suggest that the expansion of C4 ecosystems took place rapidly between 7 and 5 Myr ago in both the New World and the Old World. Synchronous expansion of C4 ecosystems in both the New World and the Old World suggests a change in global conditions, rather than local development and gradual expansion around the world. We suggest that the change may have been due to a decrease in atmospheric  $\text{CO}_2$  levels. The C4 pathway is an adaptation to reduce photorespiration that occurs in C3 plants. Under conditions of increased  $\text{CO}_2$ , C3 photosynthesis is more efficient than C4 or CAM photosynthesis<sup>4</sup>. But when the  $\text{CO}_2$  concentration in intercellular gases in C3 plants falls below about 400 p.p.m.v., the  $\text{CO}_2/\text{O}_2$  ratio decreases sufficiently to increase photorespiration rates, reducing the inherent advantage of C3 plants over C4 plants in carbon fixation<sup>4</sup>. At sufficiently low concentrations of atmospheric  $\text{CO}_2$ , combined with high temperatures and/or moisture stress, C4 plants are more efficient than C3 plants<sup>4</sup>. Therefore a rapid global expansion of C4 ecosystems may have been a result of atmospheric  $\text{CO}_2$  falling

below a critical threshold for efficient C3 photosynthesis, probably around 400 to 500 p.p.m.v. Previous studies on the palaeosols indicates that the atmospheric  $\text{CO}_2$  during the Miocene and Pliocene was less than 800 p.p.m.v. (ref. 29). The midpoint of this rapid change in global terrestrial ecosystems, about 6.3 Myr, is also a period when the carbon isotope composition of the oceans changed significantly<sup>30-32</sup>. An increase the proportion of C4 biomass or a decrease in the total terrestrial biomass would result in an isotope shift in the direction observed in the oceans. The near-temporal coincidence in the changes in the isotope composition of the terrestrial and marine carbon pools may be further evidence that a critical threshold was passed that affected the carbon budget of the biosphere.

Previously we attributed the dramatic shift in  $\delta^{13}\text{C}$  of palaeosol carbonate to an intensification of the Asian monsoon<sup>12</sup>, based in part on the less dramatic  $\delta^{18}\text{O}$  shift in the isotope composition of palaeosol carbonate that slightly preceded the  $\delta^{13}\text{C}$  shift. It now appears that the changes observed in the sediments of the Siwaliks are related to global changes in both the carbon budget and the meteoric water cycle, which do not preclude a link to the Asian monsoon system.

The modern global ecosystem includes tropical savannas, temperate grasslands, and semi-desert shrublands that have abundant C4 biomass. Our results indicate that C4 plants underwent significant expansion starting about 7 Myr ago in both the Old World and the New world. Although our results do not preclude C3 grasslands before 7 Myr ago, modern C3 grasslands are restricted to regions with cool growing seasons, such as montane or boreal conditions, or when the growing season is in the spring or winter (as in the Mediterranean climate). It is possible that C3 temperate and tropical grasslands could have existed under  $\text{CO}_2$  conditions significantly higher than modern values. However, modern ecosystems with significant C4 biomass seem to be a feature of the late Neogene to present. □

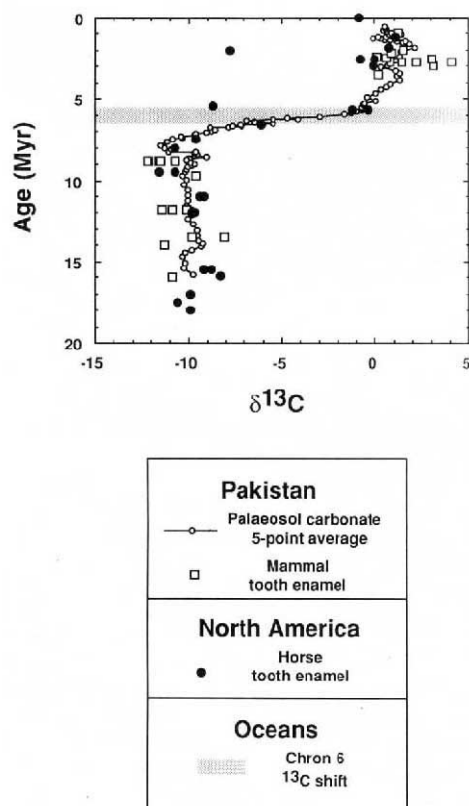


FIG. 1 Isotope transitions in North America and in Pakistan and the late Miocene carbon isotope shift in the oceans. Palaeosols from Pakistan (5-point running average shown as connected circles)<sup>12</sup>, tooth enamel from Pakistan (squares; ref. 16, and our unpublished results) and North America (filled circles). Each shows a 10–12‰ shift between 7 and 5 Myr. The age of the marine carbon shift<sup>30-32</sup> is shown as a dotted area.

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- Deines, P. in *Handbook of Environmental Isotope Geochemistry*, 1. The Terrestrial Environment (eds Fontes, J. C. & Fritz, P.) 329–406 (Elsevier, Amsterdam, 1980).
- O'Leary, M. H. *Bioscience* **38**, 325–326 (1988).
- Farquhar, G. D., Ehleringer, J. R. & Hubik, K. T. *An. Rev. Plant Physiol. Plant molec. Biol.* **40**, 503–537 (1989).
- Ehleringer, J. R., Sage, R. F., Flanagan, L. B. & Pearcy, R. W. *Trends Ecol. Evol.* **6**, 95–97 (1991).
- Smith, B. N. in *CRC Handbook of Biosolar Resources* (ed. Zaborsky, O. R.) 99–118 (CRC Press, Baton Rouge, 1982).
- Cerling, T. E., Quade, J. & Bowman, J. R. *Nature* **341**, 138–139 (1989).
- Quade, J., Cerling, T. E. & Bowman, J. R. *Geol. Soc. Am. Bull.* **101**, 464–475 (1989).
- Sullivan, C. H. & Krueger, H. W. *Nature* **292**, 333–335 (1981).
- Lee-Thorp, J. A. & van der Merwe, N. J. *South Afr. Jour. Sci.* **83**, 712–713 (1987).
- Cerling, T. E. & Hay, R. L. *Quat. Res.* **25**, 63–78 (1986).
- Cerling, T. E., Bowman, J. R. & O'Neil, J. R. *Palaeogeogr. Palaeoclimat. Palaeoecol.* **63**, 335–356 (1988).
- Quade, J., Cerling, T. E. & Bowman, J. R. *Nature* **342**, 163–165 (1989).
- Cerling, T. E. *Palaeogeogr. Palaeoclimat. Palaeoecol.* **97**, 241–247 (1992).
- Lee-Thorp, J. A., van der Merwe, N. J. & Brain, C. K. *J. hum. Evol.* **18**, 183–190 (1989).
- Thackerey, J. F. et al. *Nature* **347**, 751–753 (1990).
- Quade, J. et al. *Chem. Geol.* **94**, 183–194 (1992).
- Koch, P. L., Zachos, J. C. & Gingerich, P. D. *Nature* **358**, 319–322 (1992).
- Cerling, T. E. *Am. J. Sci.* **291**, 377–400 (1991).
- Ehleringer, J. R., Field, C. B., Lin, Z. F. & Kuo, C. Y. *Oecologia* **70**, 520–526 (1986).
- Ehleringer, J. R. & Cooper, T. A. *Oecologia* **76**, 562–566 (1988).
- Delucia, E. H., Schlesinger, W. H. & Billings, W. D. *Ecology* **69**, 303–311 (1988).
- Birkeland, P. W. *Soils and Geomorphology* (Oxford Univ. Press, New York, 1984).
- Jenny, H. *The Soil Resource* (Springer, Berlin, 1980).
- Barry, J. C., Lindsay, E. H. & Jacobs, L. L. *Palaeogeogr. Palaeoclimat. Palaeoecol.* **37**, 95–130 (1982).
- Flynn, L. J. & Jacobs, L. L. *Palaeogeogr. Palaeoclimat. Palaeoecol.* **33**, 129–138 (1982).
- Barry, J. C., Johnson, N. M., Raza, S. M. & Jacobs, L. L. *Geology* **13**, 637–640 (1985).
- Barry, J. C. & Flynn, L. J. in *European Neogene Mammalian Chronology* (ed. Lindsay, E. H.) 557–571 (Plenum, New York, 1990).
- MacFadden, B. J. *Fossil Horses: Systematics, Paleobiology and Evolution of the Family Equidae* (Cambridge Univ. Press, New York, 1992).
- Cerling, T. E. *Global Biogeochem.* **6**, 307–314 (1992).
- Keigwin, L. D. *Earth planet. Sci. Lett.* **45**, 361–382 (1979).
- Hodell, D. A. & Kennett, J. P. *Paleoceanography* **1**, 285–311 (1986).
- Hodell, D. A., Benson, R. H., Kennett, J. P. & Bied, K. R. *Paleoceanography* **4**, 467–482 (1989).

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# Revised phylogeny of whales suggested by mitochondrial ribosomal DNA sequences

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**LIVING cetaceans are subdivided into two highly distinct suborders, Odontoceti (the echolocating toothed whales) and Mysticeti (the filter-feeding baleen whales), which are believed to have had a long independent history. Here we report the determination of DNA sequences from two mitochondrial ribosomal gene segments (930 base pairs per species) for 16 species of cetaceans, a perissodactyl and a sloth, and construct the first phylogeny for whales and dolphins based on explicit cladistic methods. Our data (and earlier published myoglobin sequences) confirmed that cetaceans are closely related to artiodactyls and that all families and superfamilies of cetaceans are monophyletic. A surprising finding was that one group of toothed whales, the sperm whales, is more closely related to the baleen whales than to other odontocetes. The common ancestor of baleen whales and sperm whales might have lived only 10–15 million years ago. The suggested paraphyly of toothed whales has many implications for classification, phylogeny and our understanding of the evolutionary history of cetaceans.**

The transition from a terrestrial to the fully aquatic lifestyle of cetaceans required a complete remodelling of many biological systems. Understanding of the origin and evolutionary relationships of cetaceans was hindered by their highly specialized morphology. A link between cetaceans and ungulates was first suggested more than a hundred years ago<sup>1</sup> and recently confirmed by palaeontological<sup>2–5</sup> and molecular data<sup>6–8</sup> hinting at a closer relationship of cetaceans with artiodactyl than with perissodactyl ungulates. Living cetaceans have been assigned to the suborder Mysticeti (11 species) and the suborder Odontoceti (about 67 species), both thought to have separated from the extinct suborder Archeoceti more than 35–40 million years ago<sup>9</sup>. Fossils of the extant whales are much younger<sup>9,10</sup>. The fossil record of cetaceans is incomplete and has not provided clear evidence on the origin of and the relationships among the three suborders of cetaceans (reviewed in refs 9, 10). The taxonomic distinction between odontocetes and mysticetes seems well supported by morphological differences, which include the presence of teeth in the former and baleen, or whalebone in the latter. The ancient origin of baleen whales from a lineage of toothed whales was postulated because teeth are found in the condylarth mesonychids, an extinct group of ungulates linked to the origin of cetaceans<sup>2–5</sup> and because of the embryological potential of mysticetes to express teeth<sup>2,10</sup>.

A strongly supported phylogeny, based on cladistic methods, of the major lineages of living cetaceans is currently not available. We present a phylogenetic hypothesis for all major groups of cetaceans (except river dolphins) based on mitochondrial ribosomal DNA sequences. These genes have been used successfully in a range of molecular phylogenetic studies (see for example refs 11–14). Using the polymerase chain reaction (PCR) we amplified and directly sequenced portions of the two (12S and 16S) mitochondrial ribosomal genes for 16 species of cetaceans, the cow (an artiodactyl)<sup>15</sup>, the wild ass (a perissodactyl), a human<sup>16</sup> and a sloth. Data were analysed by maximum

parsimony<sup>17</sup>, neighbour-joining<sup>18</sup> and maximum likelihood<sup>19</sup> methods, and the robustness of the phylogenetic hypotheses tested by bootstrapping<sup>20</sup>. Edentata (sloths, anteaters and armadillos) are the most primitive order of eutherian mammals (reviewed in ref. 21) and our sloth sequences were therefore used as root in the phylogenetic analyses.

These analyses resolved some open questions in cetacean systematics with confidence (Fig. 1). All families and superfamilies of cetaceans were found to be monophyletic: the beaked whales (Ziphiidae), the baleen whales (family Balaenopteridae), the sperm whales (Physeteroidea), the Delphinoidea, the dolphins (Delphinidae) and the porpoises (Phocoenidae) (Fig. 1). In agreement with previous morphological and molecular hypotheses<sup>4–7</sup>, we find that cetaceans appear to be more closely related to artiodactyls than to perissodactyls (node a, Fig. 1). The monophyly of cetaceans is also strongly supported (node b, Fig. 1). Within cetaceans, the phylogenetic position of beaked whales must remain tentative because their placement as the sister group to all living whales is somewhat uncertain because of a low bootstrap value (node c, Fig. 1). In some of the analyses, beaked whales are placed with the sperm plus baleen whale group. We find strong support for the monophyly of the Delphinoidea (node e, Fig. 1) and the Delphinidae (node not labelled in Fig. 1). The relationships among the three families of the Delphinoidea are not well resolved, yet porpoises appear to be closer to dolphins than to belugas (node f, Fig. 1). The phylogeny within the Delphinidae remains largely unresolved (at the 50 per cent majority-rule bootstrap level). The confirmation of groupings based on previous palaeontological and molecular data raises confidence in the reliability of the phylogenetic information contained in these molecules.

A surprising outcome is the suggested sister group relationship between sperm whales and baleen whales. Sperm whales appear to be more closely related to baleen whales than they are to any other group of toothed whales (node d, Fig. 1). This phylogenetic hypothesis is at odds with the traditional odontocete-mysticete division<sup>9</sup>. The suggested paraphyly of odontocetes, making them an unnatural (not monophyletic) group, would seem to call for a reclassification of cetaceans. The taxonomic rank of baleen whales would need to be lowered if one subscribes to the cladistic view that groups of organisms that are given equal taxonomic ranks (that is, the suborders Mysticeti and Odontoceti) must each include an ancestor and all of its descendants. Evolutionary taxonomists, however, attempt to portray in the classification not only the branching of lineages but also their subsequent morphological divergence. No consensus has been reached among the schools of cladistic and evolutionary classification on this question.

More important than the potential impact on the classification of whales are the many biological and evolutionary implications of this finding. The mode and rate of morphological evolution might have proceeded differently than was previously assumed. For example, echolocation is believed to occur in all toothed whales but not in baleen whales. If our phylogenetic hypothesis is correct, it is more parsimonious to postulate that baleen whales lost the capacity for echolocation (and other behavioural, physiological and morphological adaptations related to acquisition of food), rather than to assume that they never evolved it.

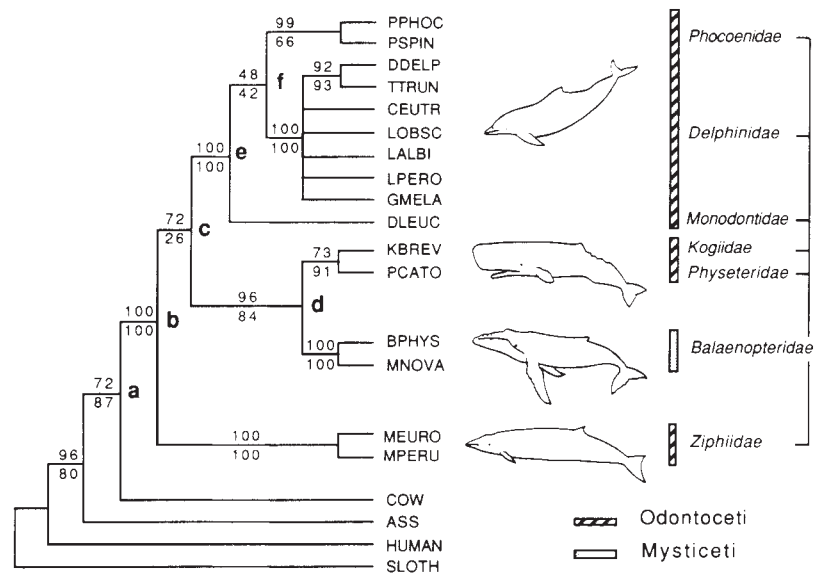
Interestingly, Schlötterer *et al.*<sup>22</sup> found surprisingly low amounts of sequence divergence (3.2%) in DNA sequences of a nuclear simple-sequence locus between the odontocetes and mysticete balaenopterids; a very low divergence for an estimated split of 35–40 Myr<sup>9,10</sup>. They presented two alternative explanations<sup>22</sup> for their finding: (1) whale DNA evolves at the slowest reported substitution rate for neutral nucleotide positions or (2) a re-evaluation of the age of modern whales is in order. On the basis of these nuclear DNA data Schlötterer *et al.*<sup>22</sup> suggest that the origin of all modern whales might date back to only 20–25 Myr and that whales might still have a much slower mutation rate than primates. Obviously, if whales had typical substitution

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FIG. 1 Majority rule bootstrap consensus tree based on 394 base pairs of the small (12S) and 536 bp of the large (16S) mitochondrial ribosomal genes of 16 cetacean species. We sequenced PPHOC; *Phocoena phocoena* (harbour porpoise); PSPIN, *Phocoena spinipinnis* (Burmeister's porpoise); DDELP, *Delphinus delphis* (common dolphin); TTRUN, *Tursiops truncatus* (bottlenose dolphin); CEUTR, *Cephalorhynchus eutropia* (black dolphin); LOBSC, *Lagenorhynchus obscurus* (dusky dolphin); LALBI, *Lagenorhynchus albirostris* (white-beaked dolphin); LPERO, *Lissodelphis peronii* (southern right whale dolphin); GMELA, *Globicephala melas* (long-finned pilot whale); DLEUC, *Delphinapterus leucas* (beluga); KBREV, *Kogia breviceps* (pygmy sperm whale); PCATO, *Physeter catodon* (sperm whale); MEURO, *Mesoplodon europaeus* (Gervais' beaked whale); MPERU, *Mesoplodon peruvianus* (peruvian beaked whale); BPHYS, *Balaenoptera physalus* (fin whale); MNOVA, *Megaptera novaeangliae* (humpback whale); and two outgroup species, wild Ass (the kulan: *Equus hemionus*) and a sloth (*Choloepus didactylus*). Tissue samples were obtained from stranded or by-catch animals, or from delphinaria. Sequences were aligned with CLUSTAL<sup>26</sup> and secondary structure models of the cow ribosomal genes<sup>27,28</sup>. For the fin whale, the 16S sequence is from ref. 24; for the 12S ribosomal gene, one position (201 in our sequence) differs from ref. 24. DNA was extracted from blood, skin, spleen or liver tissue from frozen or specimens preserved in dimethyl sulphoxide. The primers used for the PCR amplification and direct sequencing<sup>29,30</sup> of part of the 12S gene were modified<sup>31</sup> L1091 (ref. 29) and H1478 (ref. 29) primers, and those for the 16S gene were 16sar-L and 16sbr-H<sup>31</sup>. Single-stranded amplifications were done directly from whole genomic DNA, followed by three phenol-chloroform extractions and two ethanol precipitations; further details of the protocol have been reported<sup>29-31</sup>. Most of the sequences were determined (at IRBHN) with an automatic sequencer (Applied Biosystems 373A) following the manufacturer's protocols. In all cases both strands were sequenced and some species were sequenced manually as well as automatically. Sequences have been deposited in EMBL under accession numbers Z18632-Z18666 and can also be obtained from the authors. Numbers indicate bootstrap values; above the branches from a neighbour-joining<sup>18</sup> (NJ) analysis (1,000 replications; program from T. S. Whittam), below the branches numbers from a parsimony bootstrap analysis (PAUP<sup>17</sup>, 100 replications). The sequence divergences were corrected for multiple substitutions according to the observed threefold higher frequency of transitions over transversions (TS/TV), based on regression analyses done among delphinids, delphinoids and all cetaceans under 8% uncorrected sequence divergence. All regression analyses yielded slopes between 2.9 and 3.5, hence the use of a 1:3 TS/TV in NJ and parsimony analyses. A similar TS/TV for the same genes has been found among some groups of ungulates<sup>13</sup>. Three NJ analyses were performed with combined data sets and distance matrices calculated based on Kimura, maximum likelihood (PHYLP, Version 3.4.1, DNADIST<sup>19</sup>) or Jukes-Cantor distances. All three distance matrices yielded NJ trees in agreement with the topology shown here. In all analyses (NJ and parsimony) nodes a, b, d and e are stable and occur in all most parsimonious trees and in the bootstrap trees. Parsimony analyses with a TS/TV 1:1, 1:10 and transversion parsimony also yielded congruent topologies to the bootstrap tree shown underscoring the robustness of the



result. For example, in an unweighted parsimony analysis, six equally shortest trees were found (congruent with the topology shown here), tree length = 822, CI = 0.66, CI excluding uninformative characters = 0.54. In all parsimony analyses the following options were used: heuristic search (for bootstrapping, branch-and-bound for most other searches), MULPARS option in effect, MAXTREES = 100, and TBR branch swapping. Indels were coded as single characters, irrespective of their length. When indels of different length overlapped each size class of indel was assigned a particular character state. Further variations of the analyses were to use the cow as closer outgroup instead of the sloth, to include and exclude gaps, to include and exclude compensatory changes in the putative stems, and to analyse 12S, and 16S separately. All variations always yield a congruent topology with the one shown here. Nodes c and f are unstable, having low bootstrap values, and hence are regarded as tentative. In some parsimony analyses beaked whales are grouped with the sperm-baleen whale group. Node f was the only unstable node when data were submitted to the successive approximation approach to character-weighting of Farris. None of the most parsimonious solutions ever contained a monophyletic Odontoceti group. Constraining Odontoceti monophyly produced a solution eight steps longer than the shortest trees (tree length = 461 steps, bovine as outgroup, unweighted). Bootstrap analysis (NJ: 1,000 replications and parsimony: 100 replications) yielded 0% support for the monophyly of odontocetes. With parsimony analysis node d is more strongly supported by the 16S data (bootstrap value of 95%) than by the 12S data (60%), and then by both genes combined (84%). Assigned branch lengths (ACCTRAN option in PAUP, unweighted) between the major nodes are: a-b: 38, b-c: 16, c-d: 17, c-e: 24, e-f: 11. A maximum likelihood analysis (DNAML<sup>19</sup>) produced a topology in agreement with the one shown, with all branch lengths significantly positive ( $P < 0.01$ ) except those among delphinids.

rates, the estimated age of divergence would have been even younger. According to our data, baleen whales do not appear to have experienced a long independent history from toothed whales and might indeed be even younger than had been suggested by Schlötterer *et al.*<sup>22</sup>. The transversion divergence rate for mitochondrial ribosomal genes of ungulates has been calibrated to be about 0.14% per million years<sup>12,23,24</sup>. We find a 1.5 per cent transversion divergence between sperm and baleen whales. If, *prima facie*, cetaceans have a similar molecular divergence rate as ungulates then the split between sperm whales and Balaenopteridae might be only around 10–13 Myr. This is not in disagreement with the palaeontological data because the oldest fossils of balaenopterids are from Late Miocene deposits (5–10 Myr)<sup>9</sup>. The distance matrix of Schlötterer *et al.*<sup>22</sup> does not seem to rule out a close relationship of sperm whales with baleen whales. Of all groups of odontocetes, the sperm whales have the shortest mean distance to baleen whales (2.5%) (beaked (3.0%), dolphins (3.4%), porpoise (3.5%)). Furthermore, diver-

gences among families of odontocetes are higher than 2.5%. But these values are quite similar and based on a relatively small number of substitutions. Our mitochondrial data agree with these nuclear data<sup>22</sup> in suggesting the possible need for a re-evaluation of the age of modern whales, and go further to suggest that odontocetes might be paraphyletic and that baleen whales and sperm whales share a recent common ancestor.

Further support for the sperm plus baleen whale relationship derives from other nuclear data. Published myoglobin amino-acid sequences of cetaceans (10 species), cow, horse, hedgehog and human were phylogenetically analysed (PAUP<sup>17</sup>). The monophyly of the superfamilies sperm whales, beaked whales, dolphins plus porpoises (no white whale sequence is available) is supported (Fig. 2). The phylogeny is weaker<sup>20</sup>, but largely the same as the one based on our mitochondrial ribosomal sequences: sperm and baleen whales are grouped together (Fig. 2). Previous analyses<sup>25</sup> of cetacean relationships based on myoglobin and haemoglobin sequences gave equivocal results

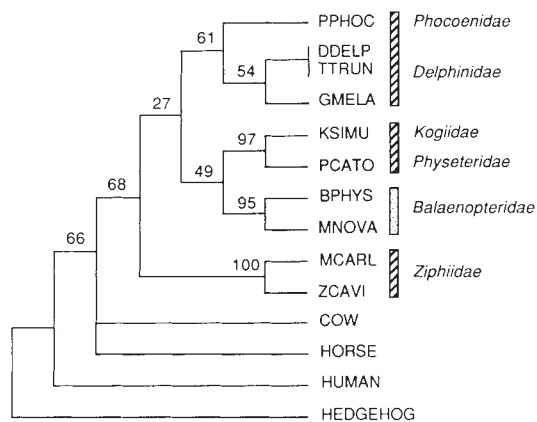


FIG. 2 Bootstrap consensus tree based on published myoglobin sequences. The majority rule bootstrap consensus tree favours the grouping of the Ziphiidae with the sperm whale-baleen whale clade at a bootstrap value of 36%. Numbers represent bootstrap values (100 replications). Analysis was done with PAUP<sup>17</sup> using a stepmatrix (PROTPARS) which gives the minimum number of amino-acid replacement substitutions needed to convert one amino acid to another. Abbreviations of the species names are as in Fig. 1, except MCARL, *Mesoplodon carlhubbsi* (Hubbs' beaked whale); ZCAVI, *Ziphius cavirostris* (Goose-beaked whale). Species were chosen to be as close as possible to the ones used for our mitochondrial sequence analysis. The inclusion of other cetacean species for which myoglobin sequences are available (*Balaenoptera acutorostrata*, *B. borealis*, *Eschrichtius gibbosus*, *Inia geoffrensis*, *Phocoena dalli*, *Stenella attenuata*, *Orcinus orca*) and further outgroups (sheep and red deer) did not change the topology but tended to lower the bootstrap values.

but hinted that toothed whales might not be monophyletic. We anticipate that more molecular data will be collected that will test the phylogeny of cetaceans. □

## Colour is what the eye sees best

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It has been argued by Watson, Barlow and Robson<sup>1</sup> that the visual stimulus that humans detect best specifies the spatial-temporal structure of the receptive field of the most sensitive visual neurons. To investigate 'what the eye sees best' they used stimuli that varied in luminance alone. Because the most abundant primate retinal ganglion cells, the P cells, are colour-opponent<sup>2-4</sup>, we might expect that a coloured pattern would also be detected well. We generalized Watson *et al.*'s study<sup>1</sup> to include variations in colour as well as luminance. We report here that our best detected coloured stimulus was seen 5–9-fold better than our best luminance spot and 3–8-fold better than Watson's best luminance stimulus. The high sensitivity to colour is consistent with the prevalence and high colour contrast-gain of retinal P cells, and may compensate for the low chromatic contrasts typically found in natural scenes.

Observers (indicated by their initials) fixated the centre of a bright, uniform yellow field (Fig. 1a) which was briefly modulated in luminance or colour. The small test spot fell on the central fovea where there are only long-wave (L) and middle-wave (M) cones<sup>5</sup>. The field was composed of red, green and yellow monochromatic lights<sup>6</sup>. By adjusting the amplitude ratio of incremental and decremental red and green components in the flash, the flash could be made to stimulate the L and M cones in any desired ratio, positive or negative.

To compare sensitivity for luminance and chromatic stimuli one must express the intensities of the stimuli in commensurate units. We did this by calculating the effect of the test flash on the L and M cones, expressed as relative modulation or contrast,  $\Delta L/L$  and  $\Delta M/M$ , in which the numerator represents the change in cone quantal catch produced by the test, and the denominator represents the cone quantal catch produced by the background. Each flash is represented as a vector in the cone contrast coordinates of Fig. 1b. Flashes on the horizontal axis stimulate L cones alone (the M cones are silenced), whereas flashes on the vertical axis stimulate M cones alone. Figure 1b depicts luminance and

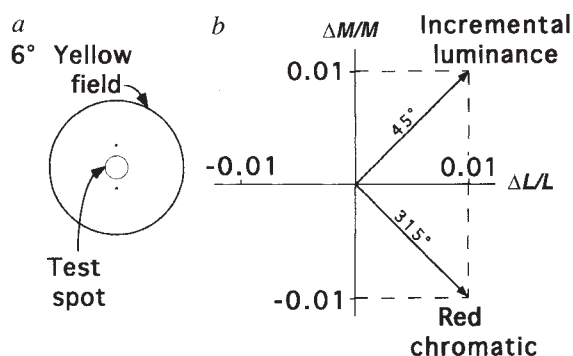


FIG. 1 a, The small test flash is presented in the centre of a bright, uniform yellow field (~3,000 trolands, metameric with 580 nm). Fixation is guided by the two black points. Stimuli were seen in a monocular maxwellian view, designed largely to correct for the eye's chromatic aberrations<sup>6</sup>. b, Luminance and red chromatic test vectors of equivalent magnitude in the cone contrast coordinates,  $\Delta M/M$ ,  $\Delta L/L$ . The luminance and chromatic flashes produce equal contrasts in the L and M cones, with the M cone contrast inverted for the chromatic flash. Luminance increments and decrements have vector angles of 45 and 225°; green and red chromatic tests have angles of 135 and 315°. The luminance flash conventionally has contrast  $c = \Delta \text{luminance}/\text{mean luminance}$ , and  $c = \Delta L/L = \Delta M/M$ .

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1. Flower, W. H. *Proc. zool. Soc. Lond.* **1883**, 466–513 (1883).
2. Van Valen, L. *Bull. Am. Mus. nat. Hist.* **132**, 1–126 (1966).
3. Szalay, F. S. *Am. Mus. nat. Hist. Nov.* **2361**, 1–26 (1969).
4. Gingerich, P. D., Wells, N. A., Russell, D. E. & Shah, S. M. I. *Science* **220**, 403–406 (1983).
5. Gingerich, P. D., Smith, B. H. & Simons, E. L. *Science* **249**, 154–157 (1990).
6. Goodman, M., Czelusniak, J. & Beeber, J. E. *Cladistics* **1**, 171–185 (1985).
7. Irwin, D. M., Kocher, T. D. & Wilson, A. C. *J. molec. Evol.* **32**, 128–144 (1991).
8. Milinkovitch, M. C. *J. evol. Biol.* **5**, 149–160 (1992).
9. Barnes, L. G., Doming, D. P. & Ray, C. E. *Mar. Mammal Sci.* **1**, 15–53 (1985).
10. Barnes, L. G. & Mitchell, E. in *Evolution of African Mammals*. (ed. Maglio, V. J. & Cooke, H. B. S.) 582–602 (Harvard University Press, London, 1978).
11. Mindell, D. P. & Honeycutt, R. L. *A. Rev. Ecol. Syst.* **21**, 541–566 (1990).
12. Allard, M. W. *et al. Proc. natn. Acad. Sci. U.S.A.* **89**, 3972–3976 (1992).
13. Miyamoto, M. M., Kraus, F. & Ryder, O. A. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6127–6131 (1990).
14. Gatsely, J., Yellon, D., DeSalle, R. & Vrba, E. S. *Molec. Biol. Evol.* **9**, 433–446 (1992).
15. Anderson, S. *et al. J. molec. Biol.* **156**, 683–717 (1982).
16. Eperon, I. C. *et al. Nature* **286**, 460–467 (1980).
17. Swofford, D. L. *Phylogenetic Analysis Using Parsimony* Version 3.0s (Illinois Natural History Survey, Champaign, 1991).
18. Saitou, N. & Nei, M. *Molec. Biol. Evol.* **4**, 406–425 (1987).
19. Felsenstein, J. *PHYLP* Version 3.4.1. (University of Washington, Seattle, 1991).
20. Felsenstein, J. *Evolution* **39**, 783–791 (1985).
21. Novacek, M. J. *Nature* **356**, 121–125 (1992).
22. Schlötterer, C., Amos, B. & Tautz, D. *Nature* **354**, 63–65 (1991).
23. Kraus, F. & Miyamoto, M. M. *Syst. Zool.* **40**, 117–130 (1991).
24. Arnason, U., Gullberg, A. & Widegren, B. *J. molec. Evol.* **33**, 556–568 (1991).
25. Czelusniak, J. *et al. in Current Mammalogy* Vol. 2 (ed. Genoways, H. H.) 545–572 (Plenum, New York, 1990).
26. Higgins, D. G. & Sharp, P. M. *Gene* **73**, 237–244 (1988).
27. Gutell, R. R. & Fox, G. E. *Prog. Nucleic Acid Res.* **16** (suppl.), R175–R313 (1988).
28. Gutell, R. R., Weiser, B., Woese, C. & Noller, H. F. *Nucleic Acids Res.* **32**, 155–215 (1985).
29. Kocher, T. D. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 6196–6200 (1989).
30. Meyer, A., Kocher, T. D., Basasibwaki, P. & Wilson, A. C. *Nature* **347**, 550–553 (1990).
31. Palumbi, S. R. *The Simple Fool's Guide to PCR* (Department of Zoology, University of Hawaii, Honolulu, 1991).

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TABLE 1 Parameters of best detected luminance and chromatic stimuli

|        |     | Spot size | Duration | Contrast | Contrast energy, $\log \text{deg}^2 \text{ s}$ |
|--------|-----|-----------|----------|----------|------------------------------------------------|
| A.C.   | LUM | 10'       | 55 ms    | 0.029    | -6.00                                          |
|        | CHR | 10'       | 140 ms   | 0.0063   | -6.92                                          |
| C.F.S. | LUM | 5'        | 55 ms    | 0.067    | -5.87                                          |
|        | CHR | 15'       | 140 ms   | 0.0066   | -6.52                                          |
| R.T.E. | LUM | 5'        | 55 ms    | 0.082    | -5.69                                          |
|        | CHR | 15'       | 100 ms   | 0.0076   | -6.55                                          |
| Ref. 1 | LUM | 18'       | 50 ms    | 0.0236   | -5.60                                          |

Comparison of spatial and temporal parameters of the optimally detected luminance (LUM) and chromatic (CHR) flashes. Thresholds are specified as both contrast and contrast energy.

red chromatic stimuli of equivalent contrast. A simple sign inversion of the M cone contrast distinguishes the two test flashes. The luminance flash increases L and M contrast equally and appears as a bright yellow flash; the red chromatic flash increases L contrast and decreases M contrast equally and appears as a red shift equiluminant with the background. Thresholds were measured for the full range of test vectors, using a temporal two-interval forced-choice method that estimates the 82% detection level<sup>7</sup>.

The threshold contour is expected to reveal two linear detection mechanisms (Fig. 2a), a red-green chromatic mechanism and a luminance mechanism. The red-green mechanism responds equally and oppositely to L and M contrast, producing a slope of +1.0 (solid lines). Such a mechanism is revealed by psychophysics<sup>8-10</sup> and by measurements of macaque red-green P (ref. 11) and lateral geniculate nucleus cells<sup>12</sup>. The luminance mechanism sums the L and M contrasts of the same sign<sup>13</sup>, and thus the threshold contour has a negative slope (dashed lines) indicating the equiluminant direction, a slope not necessarily of -1.0.

Figure 2b shows thresholds for flashes of 5 and 10 minutes of arc diameter and 200 ms duration. The ratio of chromatic:luminance sensitivity (solid versus dotted arrows) is 3.1 for the 5' spot and 4.0 for the 10' spot. Contours for the other observers have red-green slopes of 1.0 and similar aspect ratios. Red and green chromatic flashes could be distinguished as accurately as the flashes were detected, further indicating that a chromatic mechanism mediates detection.

We next searched for the size and duration for optimal detection of the luminance and chromatic spots. According to Watson *et al.*<sup>1</sup>, the best-detected stimulus has the lowest contrast energy,  $E$ ,

$$E = \iint c^2(x, y, t) dx dy dt$$

where the square of the contrast,  $c$ , is integrated over the spatial and temporal dimension of the test ( $x, y, t$ ). This stimulus identifies the spatial-temporal receptive field of the most sensitive detector<sup>1,14,15</sup>. For a luminance stimulus  $c = \Delta L/L = \Delta M/M$ . For the more general case where  $\Delta L/L \neq \Delta M/M$ , the contrast energy can be specified as

$$E = \frac{1}{2} \iiint \left\{ \left( \frac{\Delta L}{L}(x, y, t) \right)^2 + \left( \frac{\Delta M}{M}(x, y, t) \right)^2 \right\} dx dy dt.$$

Figure 3a and b shows contrast energy thresholds for luminance and chromatic spots of 5, 10 and 15' as a function of duration. The lowest luminance and chromatic thresholds occur at 55 ms and 140 ms, respectively. Figure 3c shows thresholds as a function of size, with duration roughly optimal. The luminance spots have broad minima at 5 to 15', consistent with Watson *et al.*, whereas the chromatic minima are nearer 15'. Thus the chromatic pathways have greater spatial<sup>16,17</sup> and temporal

summation<sup>18,19</sup>, providing further evidence for different signal processing streams. Note that chromatic sensitivity is higher than luminance sensitivity even for stimuli matched to the optimal luminance spots (for example 10', 60 ms; Fig. 3a, b). Table 1 lists the optimal luminance and chromatic spots for three observers, along with the data of Watson *et al.* Results for the luminance spots are quite consistent for all observers. The contrast energy thresholds of the chromatic spots are 5-9-fold lower than the luminance spots, and 3-8-fold lower than the optimal drifting luminance grating of Watson *et al.* We have examined only spots; lower thresholds might be obtained with other chromatic patterns that better match the receptive fields of chromatic neurons.

We now show that the signal:noise ratio of an ensemble of retinal P cells is compatible with the high chromatic sensitivity. P cells have overlapping opponent L-centres and M-surrounds (or vice versa) of comparable weights<sup>11,20</sup>. The independent signals from many P cells may add within a detector. In detecting a red flash, for example, the detector may add signals from P cells with L-on and M-off centres, and the density of these cells in the fovea is similar to the cone density<sup>21</sup>. Thus if the retinal area per cone is  $\alpha$ ,  $N$  is the number of P cells covering an area  $A = N\alpha$ . The impulses summed from these cells over  $T$  seconds is  $NTc\gamma$ , where  $c$  is stimulus contrast and  $\gamma$  is a cell's contrast gain. The spontaneous impulse count is  $NT\rho$ , where  $\rho$  is the spontaneous rate of a single cell, and the corresponding variance is  $\sim 0.7NT\rho$  (R. M. Shapley, personal communication). When the stimulus matches the ideal detector<sup>15</sup> in  $A$  and  $T$ , the 82% detection level ( $d' = 1.29$ ) is achieved at a contrast  $\hat{c} = (1.29)(0.7NT\rho)^{1/2}/NT\gamma$  (from the relation  $d' = \text{signal}/\sigma_n$ ). The corresponding value of  $E_{\min}$  for the detector is given by

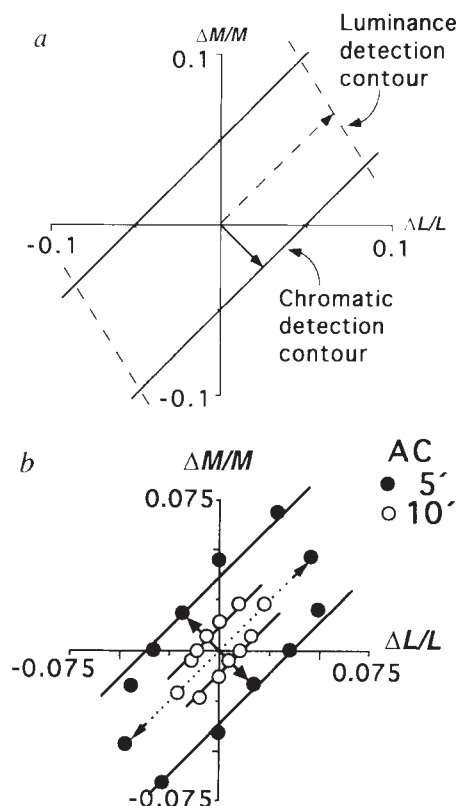


FIG. 2 a, Hypothetical threshold contours for luminance and red-green chromatic mechanisms that respond to, respectively, a linearly weighted sum and difference of the L and M cone contrasts. The dashed arrow represents a threshold-level luminance flash; the solid arrow, a threshold-level red chromatic flash. b, Threshold contours for 200 ms flashes of 5 and 10' diameter. The contours are elongated along the luminance axis, revealing greater chromatic sensitivity. The lines drawn near the data have a slope of 1.0.

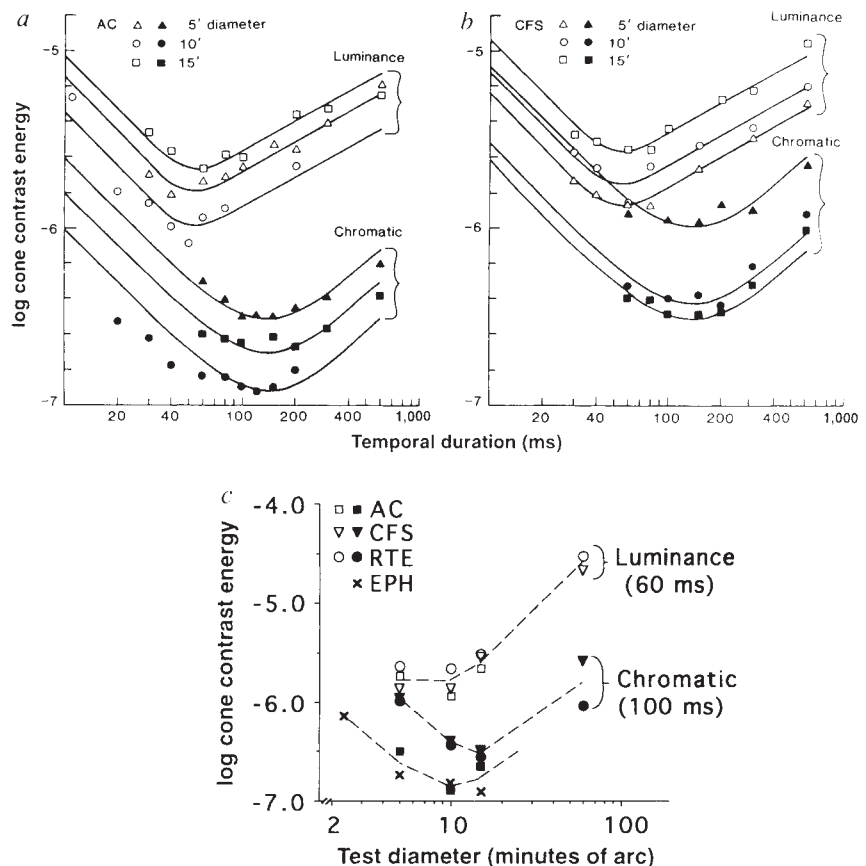


FIG. 3 *a, b*, Contrast energy thresholds (log deg<sup>2</sup> s) for test flashes of 5, 10 and 15' diameter as a function of duration. The minima estimate the optimal duration for the luminance and chromatic flashes. The fitted curves, derived from mean luminance and mean chromatic data, are of the form  $y \propto (x^{-a} + x^b)^{1/a}$ , with an asymptotic descending slope,  $-a/a = -1.0$  (Bloch's law for temporal integration) and an asymptotic ascending slope of  $b/a$  (0.56 for chromatic and 1.0 for luminance). *c*, Contrast energy thresholds as a function of flash diameter, with duration near optimal. Observer E.P.H.'s 200-ms, chromatic flashes were added for comparison.

$\epsilon^2 AT = (1.29)^2 0.7 \rho \alpha / \gamma^2$ , which is independent of both  $N$  and  $T$ . Plausible values are  $\alpha = 7 \times 10^{-5}$  deg<sup>2</sup> for the area per cone<sup>22</sup>,  $\rho = 25$  impulses per s for a cell's spontaneous rate<sup>23,24</sup> and  $\gamma = 4$  impulses per s per % contrast for a cell's contrast gain for chromatic patterns (ref. 25; R. M. Shapley, personal communication). The resulting  $E_{\min}$  of  $-7.9$  log deg<sup>2</sup> s is a log unit below our lowest chromatic energy thresholds. For a luminance stimulus, the P cell contrast gain is only  $\gamma = 0.8$  impulses per s per % contrast<sup>26</sup>, and the corresponding  $E_{\min}$  is  $-6.5$  log deg<sup>2</sup> s, about 0.6 log unit below our best luminance energy thresholds. This estimated sensitivity is probably too high because many P cells have low spatial frequency luminance attenuation<sup>27</sup> and thus would not respond optimally to the central regions of the test spot. We have not attempted similar calculations for the M cells, for it is not clear how well they are represented in the central fovea<sup>27,28</sup>. The high sensitivity to colour can thus be explained by properties of the P cells, their prevalence, high chromatic gain and noise characteristics, provided that the signals are effectively summated.  $\square$

18. Kelly, D. H. & van Norren, D. *J. opt. Soc. Am.* **67**, 1081-1091 (1977).
19. Smith, V. C., Bowen, R. W. & Pokorny, J. *Vision Res.* **24**, 653-660 (1984).
20. Reid, R. C. & Shapley, R. M. *Nature* **356**, 716-718 (1992).
21. Schein, S. J. *J. comp. Neural.* **269**, 479-505 (1988).
22. Hirsch, J. & Curcio, C. C. *Vision Res.* **29**, 1095-1101 (1989).
23. Troy, J. B. & Lee, B. B. *Invest. Ophthalmol. Vis. Sci.* **32** (suppl.), 905 (1991).
24. Crook, J. M., Lange-Malecki, B., Lee, B. B. & Valberg, A. *J. Physiol., Lond.* **396**, 205-224 (1988).
25. Shapley, R. M., Reid, R. C. & Kaplan, E. *Invest. Ophthalmol. Vis. Sci.* **32** (suppl.), 115 (1991).
26. Purpura, K., Kaplan, E. & Shapley, R. M. *Proc. natn. Acad. Sci. USA* **85**, 4534-4537 (1988).
27. Derrington, A. M. & Lennie, P. *J. Physiol., Lond.* **357**, 219-240 (1984).
28. Kaplan, E., Lee, B. B. & Shapley, R. M. in *Progress in Retinal Research* Vol. 9 (eds Osborne, N. & Chader, J.) (Pergamon, Oxford, 1990).

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## Innervation directs receptor synthesis and localization in *Drosophila* embryo synaptogenesis

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IN the *Drosophila* embryo, motor neurons form stereotyped synapses (neuromuscular junctions) on identified muscles<sup>1-3</sup>. We have used a mutant (*prospero*) that removes or delays innervation<sup>4,5</sup> to assay the role of the presynaptic motor neuron in the development of the receptive field of the postsynaptic muscle. *prospero* (*pros*) is not expressed in the muscles or their precursors. Here we find that the muscle defines the correct synaptic zone in the absence of the motor neuron by restricting putative guidance molecules to this specialized membrane region. Furthermore, the muscle expresses functional transmitter receptors at the correct developmental time without innervation. On the other hand, the muscle

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1. Watson, A. B., Barlow, H. B. & Robson, J. G. *Nature* **302**, 419-422 (1983).
2. Gouras, P. *J. Physiol., Lond.* **199**, 533-547 (1968).
3. Perry, V. H. & Cowey, A. *Vision Res.* **25**, 1795-1810 (1985).
4. Perry, V. H., Oehler, R. & Cowey, A. *Neuroscience* **12**, 1101-1123 (1984).
5. Williams, D. R., MacLeod, D. I. A. & Hayhoe, M. H. *Vision Res.* **21**, 1341-1356 (1981).
6. Cole, G. R., Stromeyer, C. F. III & Kronauer, R. E. *J. opt. Soc. Am.* **A7**, 128-140 (1990).
7. Watson, A. B. & Pelli, D. G. *Percept. Psychophys.* **33**, 113-120 (1983).
8. Noorlander, C., Heuts, M. J. G. & Koenderink, J. *J. opt. Soc. Am.* **71**, 453-459 (1981).
9. Stromeyer, C. F. III, Cole, G. R. & Kronauer, R. E. *Vision Res.* **25**, 219-237 (1985).
10. Thornton, J. E. & Pugh, E. N. Jr *Science* **219**, 191-193 (1983).
11. Lee, B. B., Martin, P. R. & Valberg, A. *J. Physiol., Lond.* **414**, 223-243 (1989).
12. Derrington, A. M., Krauskopf, J. & Lennie, P. *J. Physiol., Lond.* **357**, 241-265 (1984).
13. Stromeyer, C. F. III, Cole, G. R. & Kronauer, R. E. *Vision Res.* **37**, 1113-1137 (1987).
14. van Trees, H. L. *Detection, Estimation, and Modulation Theory* (Wiley, New York, 1968).
15. Pelli, D. G. in *Vision: Coding and Efficiency* (ed. Blakemore, C.) (Cambridge Univ. Press, Cambridge, 1990).
16. Mullen, K. *J. Physiol., Lond.* **359**, 381-400 (1985).
17. King-Smith, P. E. & Carden, D. *J. opt. Soc. Am.* **66**, 709-717 (1976).



does not localize receptors to the synapse without instruction from the motor neuron, nor does a second, much larger, synthesis of receptors occur in muscles deprived of innervation. In muscles receiving delayed innervation, or muscles innervated at aberrant synaptic sites, both receptor clustering and receptor synthesis are delayed or redirected, consistent with the new pattern of innervation. We conclude that the muscle autonomously defines the synaptic site, whereas the motor neuron directs the development of the muscle's receptive field by stimulating the synthesis and localization of transmitter receptors.

The first sign of postsynaptic neuromuscular junction (NMJ) differentiation is the expression of adhesive molecules on the muscle surface<sup>6</sup>. In some cases (for example connectin<sup>7</sup>), these molecules are initially expressed ubiquitously on the muscle and only become localized to NMJ sites during and following neuromuscular contact<sup>7</sup>. In other cases (like fasciclin III<sup>6</sup>), expression begins near the time of neuromuscular contact and is always restricted to NMJ sites<sup>6</sup>. But in both cases we find that these molecules are expressed at the correct developmental stage and localize normally to the putative NMJ sites in the absence of the motor neuron (Fig. 1). Thus, without innervation, the muscle 'knows' the location of the normal NMJ sites and is able to restrict expression of certain molecules to these specialized membrane regions; definition of NMJ sites is independent of innervation.

Fasciclin III is normally expressed at the NMJ at least through the end of embryogenesis. In aneural (*pros*) muscles, fasciclin III expression persists at the putative synaptic site for several hours, but is lost before the end of embryogenesis (data not shown). We never detected expression of fasciclin III or connectin at aberrant synaptic sites in the late embryo. Thus innervation is required to maintain a synaptic site, but is insufficient to direct the expression of these molecules.

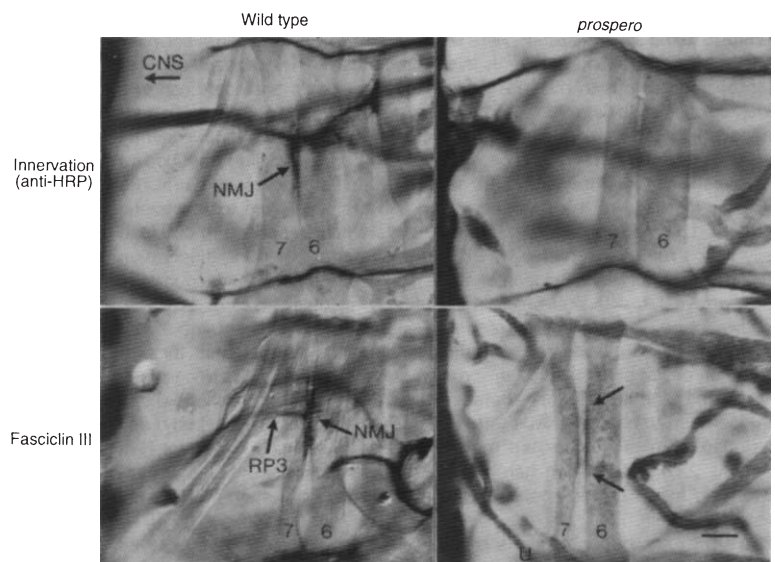
In normal *Drosophila* synaptogenesis, functional glutamate receptors (gluRs) are expressed on the muscle surface within several minutes of the initial neuromuscular contact (13–13.5 h after egg laying (AEL)) and a peak gluR density is rapidly obtained (by 14–14.5 AEL; Fig. 2). Initially, gluRs are homogeneously expressed on the muscle surface and do not cluster

at synaptic sites or elsewhere<sup>3</sup>. In *pros* mutants, this phase of gluR expression is indistinguishable from normal (Fig. 2); functional gluRs are expressed at the correct stage and increase in density with the same time course as in normally innervated muscles. Thus, the initial expression of gluRs is also innervation-independent.

By 14 h AEL, gluRs begin to localize to neuromuscular contact sites which prefigure the mature NMJs (Fig. 2); a phenomenon predominantly, perhaps exclusively, attributable to the lateral movement of previously unlocalized receptors<sup>3</sup>. In normal development, clustering progresses rapidly until gluRs are highly localized to the synaptic zone (by 16 h AEL; Figs 2 and 3). In contrast, no gluR clustering occurs in muscles lacking innervation (Figs 2, 3); the same low-density, homogenous expression of gluRs characteristic of early development persists to the end of embryogenesis. In muscles where motor neuron contact is delayed, gluR clustering at neuromuscular contact sites is similarly delayed. Likewise, in muscles with aberrant sites of neuromuscular contact, gluRs are localized to these aberrant contacts in preference to the normal synaptic zone (Fig. 3). Thus, innervation appears both necessary and sufficient to direct the localization of postsynaptic receptors to synaptic sites.

The third, and final, stage in the development of the muscle's receptive field is a large increase in gluR density at the NMJ sites (Fig. 2). This second phase of gluR appearance starts at 16–17 h AEL and continues through the end of embryogenesis (21 h AEL). Unlike the initial expression of gluRs, the second phase appears to represent the localized expression of gluRs near the NMJ as no increase in gluR density is observed in extrasynaptic sites<sup>3</sup>. This second phase of gluR expression is completely absent in *pros* mutants lacking muscle innervation (Fig. 2). But in muscles with delayed innervation, or muscles aberrantly innervated, the second phase of gluR expression is stimulated and, by the end of embryogenesis, dense concentrations of gluRs are clustered at NMJ sites (Fig. 3). Thus the second phase of gluR expression is innervation-dependent and the motor neuron is necessary and sufficient to stimulate the high expression of receptors in the muscle during late embryogenesis.

FIG. 1 *pros* encodes a nuclear protein expressed in neuronal precursors during early *Drosophila* neurogenesis<sup>4,5</sup>. Mutations in *pros* alter neuronal fates and delay or prevent pioneering of motor nerves during embryogenesis. Thus most muscles receive no innervation during the normal stages of neuromuscular synaptogenesis, although many are innervated by the end of embryogenesis. Upper panels compare patterns of innervation in late wild-type and *pros/pros* embryos (16 h AEL) in the ventral half of one abdominal (A2–A4) hemisegment; central nervous system (CNS), left; anterior, top. Scale bar, 10  $\mu$ m. Wild-type embryos have two motor nerves per hemisegment, with motor axons synapsing on all muscles by this stage<sup>1</sup>. We focus on ventral longitudinal muscles 6/7, innervated by a shared motor neuron (RP3) in the cleft between the muscles (NMJ)<sup>3,6</sup>. *pros* mutants lack motor nerves and muscles 6/7 are not innervated by RP3 or other neurons. RP3 and muscles 6/7 express fasciclin III (lower panels) from early NMJ synaptogenesis<sup>6</sup> (12–12.5 h AEL) until the end of embryogenesis (shown here 16 h AEL). In *pros* mutants where RP3 fails to innervate muscles 6/7, fasciclin III is expressed by muscles at the correct stage in a restricted region corresponding to the normal NMJ (arrows delineate extent of expression). Expression without RP3 continues for many hours (shown here 16 h AEL) but declines before the end of embryogenesis. A similar expression pattern is observed for another cell-surface protein (connectin<sup>7</sup>) in a more dorsal muscle group (not shown). METHODS. A null *prospero* mutation (*pros*<sup>14</sup>) was used<sup>4</sup>. *ru h th st p<sup>+</sup> pros*<sup>14</sup> *red e/TM3 Sb p<sup>+</sup> e* flies were outcrossed to wild-type (Oregon-R), their Sb<sup>+</sup> progeny were crossed and eggs collected. Embryos were staged



and dissected as reported earlier<sup>3</sup>. Homozygous *pros* embryos were distinguished by a grossly aberrant CNS. Embryos were stained with an anti-horseradish peroxidase antibody (Cappell), which recognizes a cell-surface antigen on all *Drosophila* neurons<sup>25</sup>. Alternatively, embryos were stained with anti-fasciclin III. Antibody staining was as previously reported<sup>3</sup>. Analyses limited to abdominal segments (A2–A6); at least 10 embryos ( $N > 100$  hemisegments) of each genotype were examined in all staining protocols.

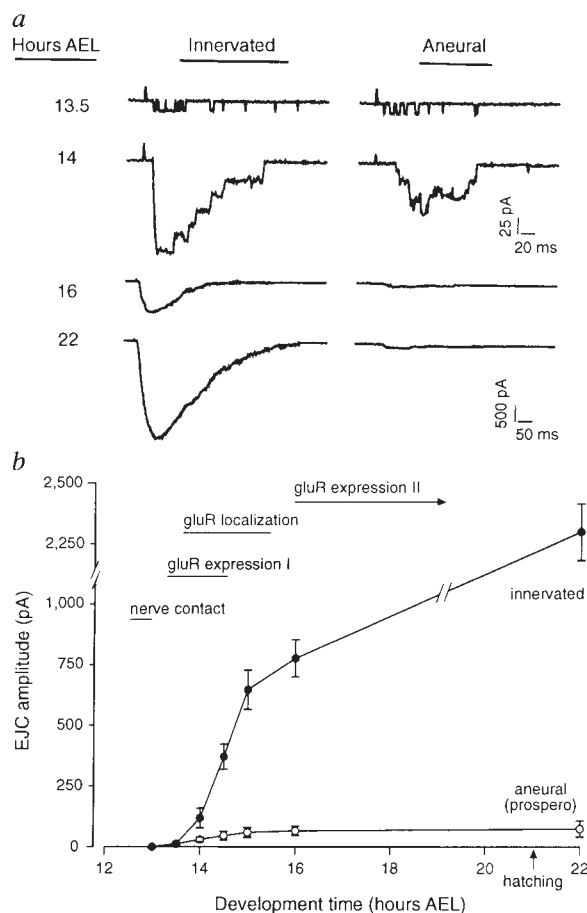
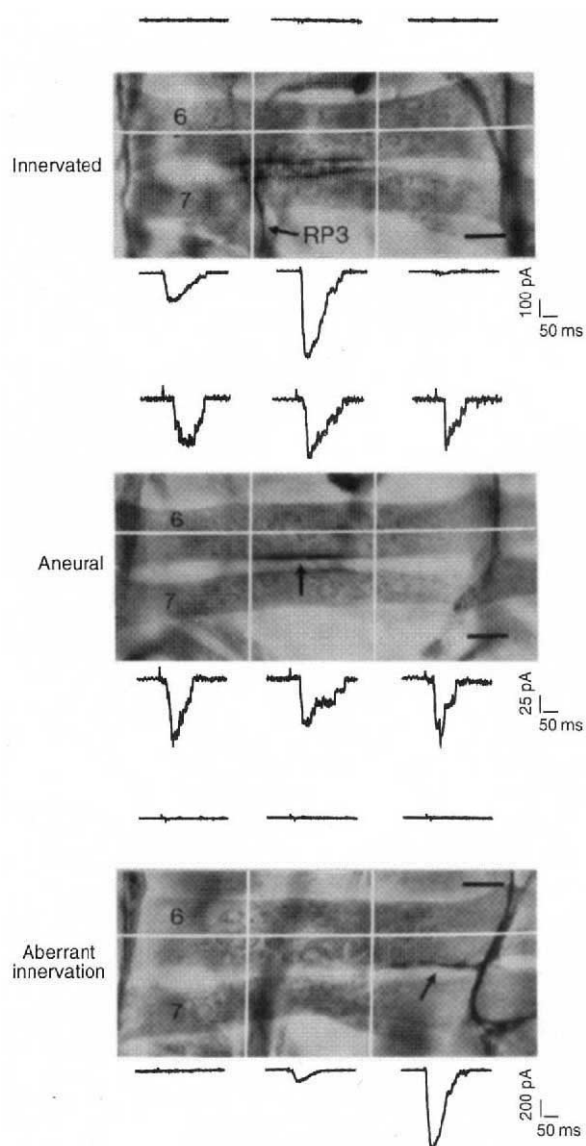


FIG. 3 During NMJ development, gluRs in muscle membrane are rapidly localized to the synaptic zone<sup>3</sup>. Here we compare gluR localization in normally innervated, aneural and aberrantly innervated muscle 6 by localized application of L-glutamate to discrete regions of the muscle surface. Each grid position on muscle 6 is associated with a current recording of the L-glutamate response in the voltage-clamped muscle; the muscle was subsequently stained with anti-horseradish peroxidase (HRP)/anti-fasciclin III antibodies to reveal sites of innervation. In normally innervated muscle, gluRs are strongly localized to the synaptic region and excluded from extrasynaptic sites by 16 h AEL (shown here); a large L-glutamate response is obtained only in the region innervated by RP3. In *pros* mutants lacking innervation (aneural muscle), no localization of gluRs occurs at the normal synaptic zone (arrow; revealed with anti-fasciclin III antibody) or elsewhere. At 16 h AEL (shown here), a uniform EJC response is observed to L-glutamate iontophoresis in all muscle regions; similar recordings are obtained through the end of embryogenesis. In *pros* mutants, muscle 6 is incorrectly innervated at a low frequency in late embryos (aberrant innervation). GluRs are localized to aberrant NMJs (arrow; revealed with anti-HRP antibody) and excluded from non-innervated muscle regions, including the normal NMJ site. At 22 h AEL (shown here), EJC amplitude indicates increased gluR synthesis has occurred at the aberrant NMJ as in later stages of normal synaptogenesis.

**METHODS.** Distribution of gluRs on the surface of muscle 6 was assayed as previously<sup>3</sup>. Muscle 6 was voltage-clamped at  $-60$  mV using standard whole-cell patch-clamp techniques. A  $0.1$  M L-glutamate solution (pH 8) was applied through pipettes ( $50$ – $75$   $\Omega$ ) to discrete domains of the muscle surface with short pulses ( $20$  ms) of negative current. Each domain of  $\sim 10 \times 10$   $\mu\text{m}$  was sampled in turn and recorded with grid position on a digital audio recorder. After recording, innervation was assayed by double staining with anti-HRP and anti-fasciclin III (see Fig. 1 legend); anti-HRP revealed innervation, anti-fasciclin III revealed normal zone of RP3 innervation. At least 5 embryos ( $N = 10$  segments) were analysed for each genotype. Scale bars,  $5$   $\mu\text{m}$ .

FIG. 2 Synaptic communication at the *Drosophila* NMJ is mediated by a large conductance L-glutamate receptor (gluR)<sup>3</sup>. Here we compare the development of the excitatory junctional current (EJC) in normally innervated and aneural (*pros*) muscle 6 by the focal iontophoresis of L-glutamate at the developing NMJ. **a**, In innervated muscle, functional gluRs are first detected at 13–13.5 h AEL (shock artefact indicates time of glutamate application). In *pros* mutants lacking innervation of muscle 6 (aneural muscle), functional gluRs are expressed at the correct stage (13.5 h AEL) and, initially, gluR density rapidly increases (14 h AEL). But gluRs fail to localize at the NMJ and, by 16 h AEL, the EJC at the innervated junction is 10-fold greater than the uninervated site. EJC amplitude in aneural muscle does not increase significantly through the end of embryogenesis. **b**, Earlier studies indicated that the initial increase in EJC amplitude occurs predominantly through the lateral movement of existing gluRs (14–16 h AEL) and latterly (16 h onward) by the increased expression of gluRs at the synaptic site<sup>3</sup>. In aneural muscle, gluRs are expressed at the correct stage, but the increase of EJC amplitude associated both with gluR localization and, later, by the increase in gluR expression at the NMJ fail to occur.

**METHODS.** Wild-type and mutant (*pros/pros*) embryos were staged and dissected as reported earlier<sup>3</sup>. Whole-cell voltage clamp ( $-60$  mV) recordings of muscle 6 were obtained using standard patch-clamp techniques<sup>3</sup>. The glutamate-gated EJC was studied by L-glutamate iontophoresis at the developing NMJ. A stock solution of  $0.1$  M L-glutamate (monosodium salt, pH 8; Sigma) was applied through pipettes ( $10$ – $20$  Megohms) with short pulses ( $20$  ms) of negative current (Farnell Pulse Generating System). Each point represents the mean peak EJC amplitude from at least 5 embryos of each genotype (mean  $\pm$  s.e.m.). Innervation was assayed with anti-horseradish immunocytochemistry (Fig. 1 legend) following recording.





The use of the *pros* mutant to remove innervation allows us to divide postsynaptic development into innervation-independent and innervation-dependent events. We find that the delineation of the synaptic zone by the localization of adhesion molecules and the initial phase of *gluR* expression are both innervation-independent. In contrast, the localization of *gluRs* to the synaptic zone and the second, localized expression of *gluRs* at the synaptic zone are both innervation-dependent. These findings strongly suggest a simple model: the muscle directs the motor neuron to the synaptic zone by means of preferential adhesion mechanisms and the motor neuron subsequently directs the localization of *gluRs* to the synaptic zone and, either directly or indirectly, stimulates the further expression of *gluRs* during later synaptogenesis.

Formative interactions during *Drosophila* neuromuscular synaptogenesis have striking parallels with synaptogenesis at the vertebrate NMJ<sup>8,9</sup>. Selective adhesion mechanisms are known to be involved in nerve-muscle recognition and control of synaptic development in several vertebrate systems<sup>10,11</sup>. Furthermore, motor neurons stimulate both receptor clustering<sup>12-14</sup> and the increased synthesis of receptors by synaptic nuclei<sup>15-17</sup> at the vertebrate NMJ. Indeed, some of the molecules involved in these interactions (such as agrin<sup>18-20</sup>) have already been identified. It appears, therefore, that synaptogenic mechanisms have been strongly conserved and it seems likely that similar molecular and genetic pathways control these interactions in *Drosophila* and other organisms. Genetic screens in *Drosophila* have already been used to identify genes that are required early in the development of the nervous system for neurogenesis<sup>21</sup> and axon guidance<sup>22</sup>, as well as others required for the proper

functioning of the mature NMJ (like ion channels<sup>23,24</sup>). This report defines an embryonic synapse in *Drosophila* which is accessible to experimental analysis. These studies can be extended, using genetic and molecular screens, to identify genes required for the construction of a synapse. □

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1. Johansen, J., Halpern, M. & Keshishian, H. *J. Neurosci.* **9**, 4318-4332 (1989).
2. Sink, H. & Whittington, P. *J. Neurobiol.* **22**, 298-311 (1991).
3. Broadie, K. & Bate, M. *J. Neurosci.* **13**, 144-166 (1993).
4. Doe, C., Chu-LaGriffa, Q., Wright, D. & Scott, M. *Cell* **65**, 451-464 (1991).
5. Vaessin, H. et al. *Cell* **67**, 941-953 (1991).
6. Halpern, M., Chiba, A., Johansen, J. & Keshishian, H. *J. Neurosci.* **11**, 3227-3238 (1991).
7. Nose, A., Mahajan, V. & Goodman, C. *Cell* **70**, 553-567 (1992).
8. Liu, D. & Westerfield, M. *J. Neurosci.* **12**, 1859-1866 (1992).
9. Dahm, L. & Landmesser, L. *J. Neurosci.* **11**, 238-255 (1991).
10. Landmesser, L., Dahm, L., Taug, J. & Rutishauser, U. *Neuron* **4**, 655-667 (1990).
11. Tosney, K., Watanabe, M., Landmesser, L. & Rutishauser, U. *Dev. Biol.* **114**, 427-452 (1988).
12. Anderson, M. & Cohen, M. *J. Physiol., Lond.* **268**, 757-773 (1977).
13. Dennis, M. A. *Rev. Neurosci.* **4**, 43-68 (1981).
14. Schuetze, S. & Role, L. A. *Rev. Neurosci.* **10**, 403-457 (1987).
15. Sanes, J. et al. *Development* **113**, 1181-1191 (1991).
16. Martinou, J.-C. & Merlie, J. P. *J. Neurosci.* **11**, 1291-1299 (1991).
17. Simon, A., Hoppe, P. & Burden, S. *Development* **114**, 545-553 (1992).
18. Nitkin, R. et al. *J. Cell Biol.* **105**, 2471-2478 (1987).
19. Reist, N., Werle, M. & McMahon, U. *Neuron* **8**, 865-868 (1992).
20. Campanelli, J., Hoch, W., Rupp, F., Kreiner, T. & Scheller, R. *Cell* **67**, 909-916 (1991).
21. Campos-Ortega, J. & Haenlin, M. *Roux's Arch. Dev. Biol.* **201**, 1-11 (1992).
22. Hortsch, M. & Goodman, C. A. *Rev. Cell Biol.* **7**, 505-557 (1991).
23. Jan, L., Jan, Y. & Hughes, H. *Cell* **69**, 715-718 (1992).
24. Salikoff, L. et al. *Trends Neurosci.* **15**, 161-166 (1992).
25. Jan, L. & Jan, Y. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2700-2704 (1982).

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## Cloning and expression of odorant receptors

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MYRIADS of odorous molecules that vary widely in structure are nevertheless readily detected and discriminated by the sense of smell, but how this is achieved by the olfactory system has been a long-standing puzzle. Several different models have been proposed<sup>1</sup>, and previous observations indicate that the recognition sites for odorous molecules could be G-protein-coupled receptor proteins<sup>2-4</sup>, an idea supported by the discovery of a new gene family that probably encodes a diversity of odorant receptors<sup>5</sup>. Here we report the identification of new members of the gene family encoding putative odorant receptors and demonstrate that they are indeed transcribed in olfactory receptor neurons. Furthermore, the receptor-encoding complementary DNA is expressed in non-neuronal surrogate cells, which generate second messenger responses upon stimulation with appropriate odorants, indicating that the receptors recognize odorants and couple to G proteins of the host cells.

We used degenerate oligonucleotides designed on the basis of the known sequences of the third and seventh putative transmembrane regions<sup>5</sup> to amplify homologous sequences from rat olfactory epithelium complementary DNA by the polymerase chain reaction (PCR). Restriction enzyme digests of the PCR products generated an array of fragments indicative of amplification of several homologous genes, confirming previous observations<sup>5,6</sup>. The PCR products were subsequently used to screen a rat olfactory cDNA library under high-stringency condi-

tions, leading to the isolation of several full-length clones. The complete nucleotide sequences of these clones were determined and the encoded protein sequence deduced (Fig. 1): they all share features of sequence and structure, including seven hydrophobic putative transmembrane domains that are common to all members of the G-protein-coupled receptor superfamily. The amino termini contain consensus sites for N-linked glycosylation, and putative phosphorylation sites are located in the third intracellular loop and the C-terminal tail. On the basis of their tissue-specific expression in the olfactory epithelium and a number of structural features, such as sequence homology and relatively short cytoplasmic loops, these receptor candidates can be considered as new members of the recently discovered putative odorant receptor family<sup>5</sup>.

To determine whether the cloned receptors are expressed in olfactory neurons, and as a first step towards a detailed mapping of the spatial distribution of receptor expression in the olfactory epithelium, we used *in situ* hybridization to probe tissue sections with a <sup>35</sup>S-labelled antisense riboprobe of the OR5 clone. Analysis of the macroscopic transcript distribution in the olfactory epithelium under high-stringency conditions (50% formamide at 55 °C) showed that all hybridization signals are restricted to the receptor cell layer in the olfactory epithelium (Fig. 2a). Analysis at a higher magnification that allowed cellular resolution confirmed that hybridizing messenger RNA is confined to receptor neurons (Fig. 2b).

Although circumstantial evidence indicates that the clones may encode odorant receptors, unequivocal proof demands that the resulting proteins should be able to interact with odorous molecules and couple to G proteins to initiate a second messenger cascade. We therefore sought a surrogate system in which the heterologous expressed receptor protein could couple to the endogenous G protein. Odorant receptors can be expressed in heterologous systems, as has been shown by injecting mRNA from olfactory tissue into *Xenopus* oocytes<sup>7</sup>. A more versatile functional assay system is required, however, for the extensive screening necessary to match a member of the large

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TABLE 1 Second messenger levels in receptor-expressing Sf9 cells

| Odour class          | OR5                              |                                               | OR12                             |                                               | Odour class                  | OR5                              |                                               | OR12                             |                                               |
|----------------------|----------------------------------|-----------------------------------------------|----------------------------------|-----------------------------------------------|------------------------------|----------------------------------|-----------------------------------------------|----------------------------------|-----------------------------------------------|
|                      | cAMP<br>(pmol mg <sup>-1</sup> ) | InsP <sub>3</sub><br>(pmol mg <sup>-1</sup> ) | cAMP<br>(pmol mg <sup>-1</sup> ) | InsP <sub>3</sub><br>(pmol mg <sup>-1</sup> ) |                              | cAMP<br>(pmol mg <sup>-1</sup> ) | InsP <sub>3</sub><br>(pmol mg <sup>-1</sup> ) | cAMP<br>(pmol mg <sup>-1</sup> ) | InsP <sub>3</sub><br>(pmol mg <sup>-1</sup> ) |
| I Fruity             |                                  |                                               |                                  |                                               | VIII Amines                  |                                  |                                               |                                  |                                               |
| Citralva             |                                  |                                               |                                  |                                               | Triethylamine                | 33.9 ± 14.8                      | 79.1 ± 17.7*                                  | 40.3 ± 6.7                       | 52.0 ± 11.4                                   |
| Citraldimethylacetal | 32.8 ± 30.4                      | 53.8 ± 9.8                                    | 42.3 ± 14.9                      | 38.8 ± 16.8                                   | Phenylethylamine             |                                  |                                               |                                  |                                               |
| II Herbaceous        |                                  |                                               |                                  |                                               | IX Organic acids             |                                  |                                               |                                  |                                               |
| Eugenol              | 36.6 ± 12.8                      | 58.8 ± 13.9                                   | 42.5 ± 14.4                      | 36.9 ± 9.7                                    | Isovaleric acid              | 28.0 ± 11.2                      | 86.5 ± 35.5**                                 | 47.5 ± 19.4                      | 48.0 ± 8.8                                    |
| Ethylvanillin        |                                  |                                               |                                  |                                               | butyric acid                 |                                  |                                               |                                  |                                               |
| III Minty            |                                  |                                               |                                  |                                               | X Sulphydryl compounds       |                                  |                                               |                                  |                                               |
| Menthone             | 40.5 ± 22.6                      | 50.0 ± 19.3                                   | 40.2 ± 27.9                      | 44.4 ± 7.9                                    | 2-Mercapthoethanol           | 32.3 ± 14.8                      | 75.5 ± 21.9                                   | 44.9 ± 22.2                      | 40.3 ± 15.0                                   |
| Eucalyptol           |                                  |                                               |                                  |                                               | Furfurylmercaptan            |                                  |                                               |                                  |                                               |
| IV Floral            |                                  |                                               |                                  |                                               | XI Methoxy pyrazines         |                                  |                                               |                                  |                                               |
| Geraniol             | 38.4 ± 21.0                      | 56.1 ± 18.9                                   | 49.4 ± 29.6                      | 43.5 ± 9.7                                    | 2-Isobutyl-3-methoxypyrazine | 33.1 ± 10.2                      | 94.6 ± 21.1**                                 | 38.6 ± 15.6                      | 48.2 ± 7.0                                    |
| Hedione              |                                  |                                               |                                  |                                               | Methoxypyrazine              |                                  |                                               |                                  |                                               |
| V Putrid             |                                  |                                               |                                  |                                               | XII Alkylpyrazines           |                                  |                                               |                                  |                                               |
| Pyrrolidine          | 39.2 ± 17.2                      | 80.4 ± 31.8*                                  | 40.8 ± 13.3                      | 43.5 ± 8.2                                    | 2,3,5-Trimethylpyrazine      | 33.8 ± 9.4                       | 66.2 ± 7.9                                    | 40.7 ± 13.3                      | 54.5 ± 14.4                                   |
| Pyrazine             |                                  |                                               |                                  |                                               | 2-Methylpyrazine             |                                  |                                               |                                  |                                               |
| Chemical groups      |                                  |                                               |                                  |                                               | XIII Stereoisomers           |                                  |                                               |                                  |                                               |
| VI Aromates          |                                  |                                               |                                  |                                               | d-Carvone                    | 29.7 ± 5.1                       | 56.7 ± 19.1                                   | 33.2 ± 8.9                       | 37.6 ± 11.8                                   |
| Coniferan            | 32.9 ± 10.1                      | 62.7 ± 7.4                                    | 42.0 ± 14.9                      | 35.1 ± 9.0                                    | L-Carvone                    |                                  |                                               |                                  |                                               |
| Helional             |                                  |                                               |                                  |                                               | control                      | 37.8 ± 14.5                      | 53.4 ± 18.0                                   | 38.1 ± 21.3                      | 42.8 ± 5.9                                    |
| VII Aldehydes        |                                  |                                               |                                  |                                               |                              |                                  |                                               |                                  |                                               |
| Lylal                | 36.2 ± 18.6                      | 119.0 ± 21.0***                               | 38.1 ± 22.3                      | 48.6 ± 8.2                                    |                              |                                  |                                               |                                  |                                               |
| Lilial               |                                  |                                               |                                  |                                               |                              |                                  |                                               |                                  |                                               |

Nomenclature of compounds not listed in the Merck Index<sup>15</sup>: citralva: 3,7-dimethyl-2,6-octadienenitrile; citraldimethylacetal: 1,1-dimethoxy-3,7-dimethyl-2,6-octadiene; lylal: 4-(4-hydroxy-4-methylpentyl)-3-cyclohexene-1-carboxyaldehyde; hedione: 3-oxo-2-pentylcyclopentaneacetic acid methyl ester; coniferan: 2-t-pentylcyclohexenol acetate; helional:  $\alpha$ -methyl-1,3-benzodioxole-5-propanol; lilial: 4-(1,1-dimethylethyl)- $\alpha$ -methylbenzenepropanal. Procedures for expressing recombinant receptor clones in Sf9-cells and isolation of membranes are described in Fig. 3 legend. Odorant-induced second messenger formation was assayed as described<sup>11</sup> using a rapid quenching device. Briefly, a sample of Sf9-cell membranes (300  $\mu$ g protein) was rapidly mixed with a buffered solution containing the appropriate mixture of odorous compounds (1  $\mu$ M each). The reaction was terminated after 1 s by injecting cooled perchloric acid (10%). Quenched samples were assayed for cAMP<sup>16</sup> and InsP<sub>3</sub> ref. 17. Data represent the mean of 3 duplicate experiments  $\pm$  s.d. Statistical significance of the data was evaluated using the unpaired *t*-test.

\* Marginally significant ( $P < 0.1$ ); \*\* significant ( $P < 0.05$ ); \*\*\* very significant ( $P < 0.005$ ).

receptor family with the appropriate ligands from the vast array of odorants.

The baculovirus-Sf9 cell system enables a variety of foreign DNAs to be expressed with high efficiency<sup>8</sup>, including G-protein-linked receptor proteins<sup>9</sup>. It can be used to couple a heterologous expressed receptor to the second messenger cascades<sup>10</sup>. We therefore infected a cell line derived from *Spodoptera frugiperda* (Sf9) with baculovirus harbouring the receptor-encoding cDNA downstream from the strong polyhedrin promoter. About 48 h after infection with the recombinant virus (OR5, OR12), the cells contained high levels of receptor-specific mRNA. Membranes from Sf9 cells prepared two days postinfection were assayed for odorant-induced generation of cyclic AMP or inositol trisphosphate (InsP<sub>3</sub>)<sup>11</sup>.

To overcome any obstacle to discovering odorants that might interact as agonists with the chosen receptors (OR5, OR12), we screened a panel of odorous compounds. Representatives of different odour classes and compounds representing different chemical groups were assayed for their potential to elicit a second messenger response (Table 1). The results indicate that most of the odorants did not affect the basal levels of either cAMP or InsP<sub>3</sub> in membrane preparations from receptor-expressing Sf9 cells. In Sf9 cells infected with recombinant OR12 virus, there was no significant change in cAMP or InsP<sub>3</sub> upon stimulation with any of the odorant mixtures. In contrast, assays of the reactivity of OR5-expressing cells towards the odorant panel revealed that in fact some odorants elicited a significant increase in InsP<sub>3</sub> concentration, more than half were inactive.

FIG. 1 Alignment of the amino-acid sequences deduced from the nucleotide sequence of five rat cDNA clones encoding putative odorant receptors. Conserved amino acids found in all of the rat receptors are marked by asterisks. Transmembrane domains are boxed (TM1-TM7). Gaps inserted for optimal alignment or missing amino acids at the N terminus are indicated by dots. Putative sites for N-glycosylation are underlined.

**METHODS.** The conserved amino-acid sequences MAYDRY-VAIC and PMLNPFYI of putative odorant receptors<sup>5</sup> were used to design degenerate oligonucleotides of the sequences 5'-ATGGC(G/C/T)TA(C/T)GA(C/T)(A/C)G(A/G/C)TA(C/T)GT(G/C)-GC(G/C/T)AT(A/T/C)TG-3' and 5'-TA(T/G/A)AT(G/A)AA(C/G)-GG(G/A)TT(T/G/C)A(G/A)CAT(T/C/G/A)GG-3', respectively. Oligonucleotides were combined in PCR reactions with single-stranded cDNA synthesized by reverse transcription of poly(A)<sup>+</sup> RNA isolated from rat olfactory epithelium<sup>18</sup>. PCR was done using *Taq* polymerase (Promega) for 38 cycles at 96 °C for 30 s, 55 °C for 2 min, and 72 °C for 3 min, with the final extension lengthened to 10 min. The amplified DNA (about 500 base pairs) was gel-excised<sup>19</sup> and cloned into M13 vectors for DNA sequence analysis by dideoxynucleotide chain termination sequencing<sup>20</sup> using T7 DNA polymerase (Pharmacia). Using a mixture of <sup>32</sup>P-labelled cloned PCR fragments coding for different putative odorant receptors as hybridization probe, several positive plaques in a screen of 1 × 10<sup>6</sup> bacteriophage from a rat olfactory cDNA library prepared

|  | TM 1                                  |                          | TM 2                       |                           |
|--|---------------------------------------|--------------------------|----------------------------|---------------------------|
|  | OR5                                   | OR12                     | OR18                       | OR37                      |
|  | MTNRQTVISQFLGLLPIPEHGH                | VYVAFISMVLTITLGLNIIITLIT | LDSHLHT                    | PMYFLSNLSFSLCFSSVTMP      |
|  | .....SVTEFLIACLTQDGLRM                | PLPFLFLGFMVTVGNLIGFLIG   | LNHLHT                     | PMYFLFLNLSVDFCFSSVTMP     |
|  | MGNNN...ITEFLIIGLTQDQGRK              | ALFVIFFLIYIVHTGNNLIVTVTI | ASPSLGS                    | PMYFLFLNLSVDFCFSSVTMP     |
|  | .....LLGLSGYFKETI                     | LYFVILVWVYLVHTGNNLIVTVTI | FDHLHT                     | PMYFLFLNLSVDFCFSSVTMP     |
|  |                                       |                          |                            |                           |
|  | TM 3                                  |                          | TM 4                       |                           |
|  | OR5                                   | OR12                     | OR18                       | OR37                      |
|  | LLQNMQSQVPSIPYAGCLSQ                  | IVYFLFFGLDGLNLLVAMAY     | DRYVAICPLHYMSIMSPK         | LCVSLVWLSVWLTTFHML        |
|  | LLQNMQNDTSITYTGCLTQ                   | MYFSMVAFMEIFLVSMAY       | DRYVAICPLHYTSIMSPK         | FCVCLGSLGWVFNVLVSM        |
|  | MLMSFISKNIISHGCMTO                    | LFYFCFVVSSETFILSAMAY     | DRYVAICPLHYTVMTSPQ         | VCLLLGLGAYVMGFSEAMA       |
|  | LIADLLYDQRTISFRACMSQ                  | LFIEHLFGGVIVILVAMAY      | DRYVAICPLHYLAIMNR          | VCLITLLIFAWTGGFTSLI       |
|  | TLVSLISKRNISFSGCTVQ                   | MEVGFAMGSTECLIGMAF       | DRYVAICPLHYSVMSKE          | VYVMSASASWFGSGINSVV       |
|  |                                       |                          |                            |                           |
|  | TM 5                                  |                          | TM 6                       |                           |
|  | OR5                                   | OR12                     | OR18                       | OR37                      |
|  | LLMARLSPCEDNVIPIHFFCDMSALLKLACSDTRVNE | VVPIFVSLFVLFPFALTIMSVV   | RIVSVILKVPSSQGIY           | KAFSSC                    |
|  | LLMARLSPCEDNVIPIHFFCDMSALLKLACSDTYNE  | LMPIFLGGLLIVIPFLLLVMTYV  | OIVCSILKVPSTRAIY           | KIFSTC                    |
|  | GLMLNLTFCADNVLNHHMCDLPLLELSCNSTFINE   | LVPIVAVIAIDVAVISIFISYA   | LLHSSILRMHSTEGRS           | KAFSTC                    |
|  | VFWVLPFCGPNVIDHIFCDMSPLLVLACTDTYFEG   | LVPIVAVIAIDVAVISIFISYA   | LLHSSILRMHSTEGRS           | KAFSTC                    |
|  | SLAMRLPFCGPNVINHTFEVLAVLKACADISINI    | VTVVSNMVAIVPLLLIFPSVY    | LLITTLIRMSASGR             | KAFSTC                    |
|  |                                       |                          |                            |                           |
|  | TM 7                                  |                          | TM 8                       |                           |
|  | OR5                                   | OR12                     | OR18                       | OR37                      |
|  | GSHLSVSLFPGTVIPVLYL                   | C.PSANNST.VK             | E...TV...MSLMTITVTPMLNPI   | YSLNRNDIKAGAMELIFCKRKIQNL |
|  | GSHLSVSLFPGTVIPVLYL                   | C.PSANNST.VK             | E...TV...MSLMTITVTPMLNPI   | YSLNRNDIKAGAMELIFCKRKIQNL |
|  | SSHLIVVCLLFGSGAPMYL                   | KLPSILPLDQCK             | ...V...SSLFTVITVTPMLNPI    | YSLNRNDIKAGAMELIFCKRKIQNL |
|  | SSHLIVVCLLFGSGAPMYL                   | R.PVNFPI.DK              | CT...TV...SSLFTVITVTPMLNPI | YSLNRNDIKAGAMELIFCKRKIQNL |
|  | SAHATVTVIPYCTIFSMYA                   | K.PKSQDLT.GK             | DKFQTSKDIISLFGVTVTPMLNPI   | YSLNRNDIKAGAMELIFCKRKIQNL |

in  $\lambda$ NM1149 were detected. Inserts of plaque-purified positive clones that showed an amplification product of the appropriate size in a PCR reaction using the degenerate primers, were subcloned into M13 vectors and sequenced using standard procedures<sup>18-20</sup>.



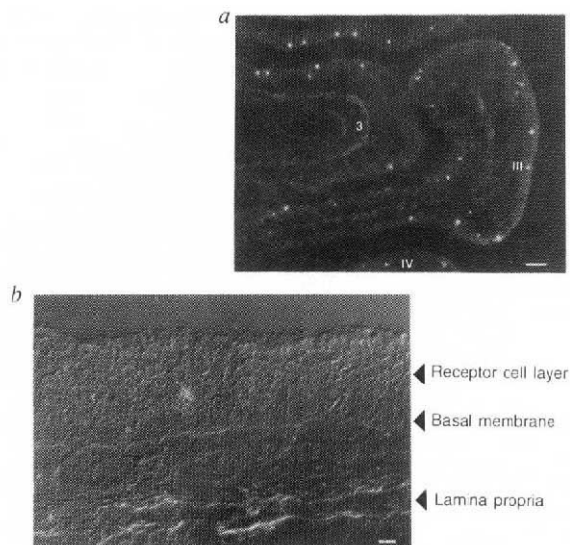


FIG. 2 Hybridization of  $^{35}\text{S}$ -labelled OR5 antisense RNA to mRNAs in coronal sections of rat olfactory epithelium. *In situ* hybridization was done under high-stringency conditions. *a*, Dark-field photomicrograph; endoturbinates (II, III, IV) and ectoturbinates (3) are indicated according to ref. 21. Scale bar, 100  $\mu\text{m}$ . *b*, Differential interference-contrast photomicrograph showing olfactory epithelium at higher magnification. Scale bar, 10  $\mu\text{m}$ .

**METHODS.** 10- $\mu\text{m}$  cryostat sections from the nasal cavities of rats were thaw-mounted on silanated slides. Radiolabelled antisense RNA probes were generated by *in vitro* transcription of the clone OR5 using the SP6/T7 transcription system (Boehringer) with SP6 polymerase in the presence of [ $\alpha$ - $^{35}\text{S}$ ]CTP. Sections were incubated overnight with hybridization solution containing 500 pg labelled antisense RNA and 50% formamide at 37  $^{\circ}\text{C}$ . After RNase treatment for 30 min, sections were washed in 0.1  $\times$  SSC at 55  $^{\circ}\text{C}$ . Slides were dipped in NTB-2 photographic emulsion (Kodak) and developed after exposure for 21 d.

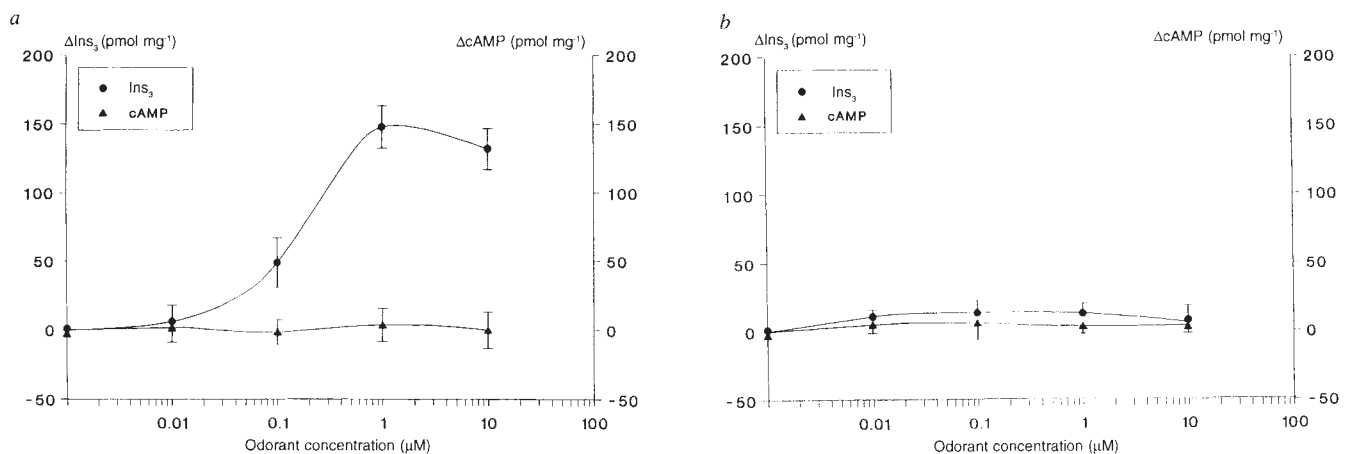


FIG. 3 Dose-response curve of odorant-induced second messenger formation in membrane preparations from Sf9 cells infected with *a*, recombinant (AcMNPV/OR5), and *b*, wild-type (AcMNPV) virus. Challenging membranes from Sf9 cells expressing the OR5 receptor with a mixture of odorants (linal, lilial) elicited a concentration-dependent generation of  $\text{InsP}_3$ ; the preparation used in this set of experiments gave a slightly higher response. There was no change in the cAMP level. Cells expressing wild-type virus gave no response (*b*). The basal level of the second messengers in the membrane preparations from Sf9 cells infected with wild-type virus was: cAMP,  $39.2 \pm 4.9$ ;  $\text{InsP}_3$ ,  $48.4 \pm 7.2$ ; from Sf9-cells infected with the recombinant (AcMNPV/OR5) virus: cAMP,  $37.8 \pm 14.5$ ;  $\text{InsP}_3$ ,  $53.4 \pm 18.0$ .

**METHODS.** A *KpnI* fragment of the full-length OR5 cDNA was used to construct the recombinant baculovirus transfer vector pVL1393/OR5. Therefore a M13 vector that contained the OR5 cDNA inserted in the *EcoRI* site was restricted with *KpnI*. Owing to a single *KpnI* restriction site 15 bases upstream of the start methionine codon in the OR5 cDNA and another in the multiple cloning site of M13 vector, a shortened OR5 *KpnI* fragment was released, which was isolated by standard procedures<sup>18,19</sup>. The baculovirus transfer vector pVL1393 was prepared by digestion with *KpnI*, resulting in the release of a small *KpnI* fragment, which was separated from the vector by electrophoresis in low-melting-point agarose and  $\text{QN}^+$  extraction<sup>19</sup> before dephosphorylation. The OR5 *KpnI* fragment was ligated into the *KpnI*-restricted and dephosphorylated pVL1393 vector, placing its transcription under the control of the strong polyhydriin promoter and initiating protein expression by a self-encoded AUG start codon. By analogy with OR5 cDNA, the full-length OR12 cDNA was also placed behind the strong polyhydriin promoter. Therefore the cDNA was isolated from a M13 plasmid

as an *EcoRI* fragment and integrated into the *EcoRI* site of the baculovirus virus transfer vector pVL1392, resulting in the recombinant baculovirus transfer vector pVL1392/OR12. Cotransfections of the transfer plasmids (pVL1393/OR5, pVL1392/OR12) with wild-type AcMNPV DNA (AcMNPV is *Autographa californica* multiple nuclear polyhedrosis virus) into Sf9 cells and purification of recombinant baculovirus (AcMNPV/OR5, AcMNPV/OR12) produced by homologous recombination were according to ref. 22. Purified recombinant virus was used to infect  $1.5 \times 10^8$  Sf9 cells in 100-ml spinner cultures at high multiplicity of infection<sup>22</sup>. Cells were collected 48 h postinfection and membrane fractions isolated as described<sup>23</sup>. Cells were pelleted (at 250g for 10 min at 4  $^{\circ}\text{C}$ ), washed with Ringer solution (120 mM NaCl, 5 mM KCl, 1.6 mM  $\text{K}_2\text{HPO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 25 mM  $\text{NaHCO}_3$ , 5 mM glucose, pH 7.4) and then disrupted in homogenization buffer (10 mM Tris-HCl, pH 8.0, 2 mM EGTA, 3 mM  $\text{MgCl}_2$ ) with antiproteases according to ref. 24 using a glass homogenizer. The homogenate was centrifuged and the pellet washed twice. Supernatants were centrifuged at 33,000g for 20 min. The final pellet was resuspended in homogenization buffer and the protein concentration determined<sup>25</sup>. Assay of odorant stimulation and second messenger concentration was as described<sup>4,11</sup>. Briefly, suspensions of Sf9 cell membrane preparations (300  $\mu\text{g}$  protein) were rapidly mixed with 'stimulation buffer' (200 mM NaCl, 10 mM EGTA, 50 mM MOPS, 2.5 mM  $\text{MgCl}_2$ , 1 mM DTT, 0.05% Na-cholate, 1 mM ATP, 1  $\mu\text{M}$  GTP and 0.02  $\mu\text{M}$  free  $\text{Ca}^{2+}$ ) containing odorants at the appropriate concentrations. The reaction was stopped after 1 s by injecting 10% perchloric acid. Quenched samples were assayed for second messenger concentrations; cAMP was determined according to ref. 16;  $\text{InsP}_3$  was measured using a receptor-binding assay<sup>17</sup>.

There was no change in the cAMP concentration. Interestingly, all the reactive odorants also induce  $\text{InsP}_3$  responses in isolated olfactory cilia<sup>12</sup>. As the odorous aldehydes (linal, linal) are the most active ligands, their effect was assayed over a wide concentration range.

The dose-response curve (Fig. 3a) indicates that infected cells respond to submicromolar concentrations of odorants with a stimulus-dependent  $\text{InsP}_3$ -signal; there is no change in cAMP. An apparent saturation is reached at about 1  $\mu\text{M}$ . Control cells, namely Sf9 cells infected with the wild-type virus, did not respond to odour stimulation (Fig. 3b). Our data indicate that the heterologous expressed OR5 receptor interacts with odorants and couples to the phosphatidylinositol system of the host cells. Thus we have shown that a rat odorant receptor can be func-

tionally expressed in these cells. The observation that the OR5 receptor does not just interact with a particular agonist but responds to several odorous compounds, although less efficiently, may indicate a relatively broad ligand specificity of odorant receptors. The failure to detect any responsiveness for the OR12 receptor could be due to a narrower tuning of this receptor type.

The expression of individual odorant receptors in surrogate cells should allow the reconstitution of the olfactory signalling cascade and so provide new insight into the specificity of ligand binding, activation of second messenger pathways and the role of receptors in signal termination<sup>13,14</sup>. Receptor-specific molecular probes can now be used to approach long-standing basic questions in olfactory signalling. □

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1. Amoore, J. E. *Chem. Senses Flavor* **2**, 267-281 (1977).
2. Pace, U., Hanski, E., Salomon, Y. & Lancet, D. *Nature* **316**, 255-258 (1985).
3. Jones, D. T. & Reed, R. R. *Science* **244**, 790-795 (1989).
4. Breer, H., Boekhoff, I. & Tareilus E. *Nature* **345**, 65-68 (1990).
5. Buck, L. & Axel, R. *Cell* **65**, 175-187 (1991).
6. Levy, N. S., Bakalyar, H. A. & Reed, R. R. *J. Ster. Biochem. molec. Biol.* **39**, 633-637 (1991).
7. Dahmen, N., Wang, H. L. & Margolis, R. L. *J. Neurochem.* **58**, 1176-1179 (1992).
8. Miller, L. K. A. *Rev. Microbiol.* **42**, 177-199 (1988).
9. George, S. T., Arbabian, M. A. & Ruoho, A. E. *Biochem. biophys. Res. Commun.* **163**, 1265-1269 (1989).
10. Parker, E. M., Kameyama, K., Higashijima, T. & Ross, E. M. *J. biol. Chem.* **266**, 519-527 (1991).
11. Boekhoff, I., Tareilus, E., Strotmann, J. & Breer, H. *EMBO J.* **9**, 2453-2458 (1990).
12. Breer, H. & Boekhoff, I. *Chem. Senses* **16**, 19-29 (1991).
13. Boekhoff, I. & Breer, H. *Proc. natn. Acad. Sci. U.S.A.* **89**, 471-474 (1992).
14. Boekhoff, I., Schleicher, S., Strotmann, J. & Breer, H. *Proc. natn. Acad. Sci. U.S.A.* **89**, 11983-11987 (1992).

15. *Merck Index* 11th edn (eds Budavari, S., O'Neil, M. J., Smith, A. & Heckelman, P.E.) (Merck, Rahway, NJ, 1989).
16. Steiner, A. L., Pagliara, A. S., Chase, L. R. & Kipnis, D. M. *J. biol. Chem.* **247**, 1114-1120 (1972).
17. Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. *Cell Signal* **1**, 147-156 (1989).
18. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* 2nd edn (Cold Spring Harbor Laboratory Press, New York, 1989).
19. Langridge, J., Langridge, P. L. & Bergquist, P. L. *Analyt. Biochem.* **103**, 264-271 (1980).
20. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
21. Liebh, H. G. *Anat. Anz.* **138**, 170-179 (1975).
22. Summers, M. D. & Smith, G. E. *Tex. Agric. exp. St. Bull.* **1555**, 1-57 (1987).
23. Wong, S. K-F., Parker, E. M. & Ross, E. M. *J. biol. Chem.* **265**, 6219-6224 (1990).
24. Dadi, H. K. & Morris, R. J. *Eur. J. Biochem.* **144**, 617-628 (1984).
25. Bradford, M. M. *Analyt. Biochem.* **65**, 248-254 (1976).

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## Benzodiazepine-induced motor impairment linked to point mutation in cerebellar GABA<sub>A</sub> receptor

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THE selectively outbred alcohol-non-tolerant (ANT) rat line<sup>1</sup> is highly susceptible to impairment of postural reflexes by benzodiazepine agonists<sup>2</sup> such as diazepam. ANT cerebella are generally devoid<sup>3</sup> of diazepam-insensitive high-affinity binding of the benzodiazepine [<sup>3</sup>H]Ro15-4513 (refs 4, 5), whereas in non-selected strains such binding marks a granule-cell-specific GABA<sub>A</sub> (γ-aminobutyric acid) receptor containing the α6 subunit<sup>5,6</sup>. A critical determinant for diazepam insensitivity of this 'wild-type' cerebellar GABA<sub>A</sub> receptor is an arginine residue<sup>7</sup> in α6 position 100, where other α subunits carry a histidine<sup>8</sup>. Here we report that the α6 gene of ANT rats is expressed at wild-type levels but carries a point mutation generating an arginine-to-glutamine substitution at position 100. In consequence, α6(Q100)β2γ2 receptors show diazepam-mediated potentiation of GABA-activated currents and diazepam-sensitive binding of [<sup>3</sup>H]Ro15-4513. Our results suggest that cerebellar motor control may be a distinct behavioural correlate of the α6-subunit-containing GABA<sub>A</sub> receptor subtype.

To search for a molecular alteration in the α6 subunit of ANT rats, complementary DNA constructed from cerebellar RNA of ANT and control animals of the alcohol-tolerant (AT) line<sup>1</sup> was amplified by polymerase chain reaction (PCR)<sup>9</sup> with α6

sequence-specific<sup>6</sup> oligonucleotide primers. Amplified DNA fragments were cloned and for each fragment, several recombinants were analysed by DNA sequencing<sup>10</sup>. This analysis revealed a difference in a single nucleotide between the ANT and wild-type α6 cDNAs (Fig. 1), which generated an amino-acid substitution in the functionally critical position 100 of the α6 subunit<sup>7</sup>; the arginine (CGA codon) in this position of the wild-type α6 subunit<sup>6</sup> is replaced by a glutamine (CAA) in the ANT-specific α6 subunit.

To determine whether this single nucleotide exchange in the cDNA results from a point mutation in the α6 gene or is introduced by RNA editing<sup>11,12</sup>, we amplified the appropriate exonic sequence from genomic DNAs isolated independently from the livers of two ANT and two control rats. DNA was also isolated from one animal in the ANT population (rat 5, Table 1), whose cerebellum gave half the level of diazepam-insensitive [<sup>3</sup>H]Ro15-4513 binding compared with normal rat cerebellar tissue. PCR-amplified DNA fragments were cloned in single-stranded form and were analysed by dual hybridization to oligonucleotides designed to detect the single nucleotide difference in the ANT and wild-type α6 subunit cDNAs. Results (Table 1) showed that all clones derived from the control genomic DNA hybridized solely with the oligonucleotide specific for the arginine (wild-type) form of α6. Conversely, all ANT-derived plaques hybridized with the probe specific for the glutamine form of α6. This indicates that the nucleotide substitution in the cDNA results from a point mutation in the α6 gene of ANT rats. In support of this conclusion, about one half of the clones derived from the genome of the pharmacologically 'atypical' ANT rat hybridized with the glutamine-specific oligonucleotide, the other half with the arginine-specific probe. Hence, pharmacologically atypical ANT rats are heterozygous for the mutation. The significance of heterozygosity regarding behavioural drug sensitivity needs further study. It appears that these atypical ANT animals (4 out of 38 ANT, F<sub>40</sub> generation; our unpublished results) may not have been selected as tightly as the homozygous mutants<sup>3</sup>.

*In situ* hybridization using an α6 subunit-specific oligonucleotide was done to determine whether α6 gene expression is



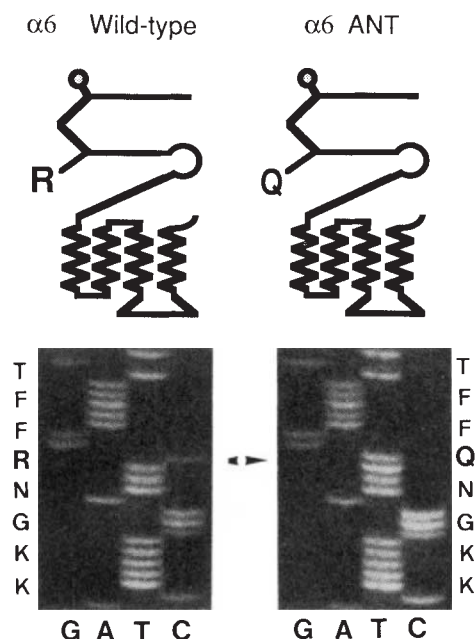


FIG. 1 Single nucleotide mutation in the  $\alpha 6$  subunit of ANT rats. The amino-acid substitution of the arginine residue (R) in wild-type  $\alpha 6$  for a glutamine (Q) in the ANT  $\alpha 6$  subunit is depicted schematically. The  $\alpha 6$  subunit has four predicted transmembrane regions and a large extracellular domain with putative *N*-linked glycosylation sites (lollipop). The single nucleotide difference (double arrow) leading to the substitution is revealed by the DNA sequence<sup>10</sup> (non-coding strand, gel lanes: G, A, T, C). The amino-acid sequences encoded by the reverse complements of the two DNA ladders and reading N- to C-terminal from top to bottom are listed next to the gel patterns.

**METHODS.** Poly(A)<sup>+</sup> RNA isolated from ANT and control rat cerebella with oligo(dT)<sub>25</sub>-tagged magnetic microspheres (Dynabeads, Dynal) was used to prepare cDNA. The entire coding region of the  $\alpha 6$  subunit was amplified as two fragments by PCR<sup>9</sup> (Vent polymerase, New England Biolabs) with oligonucleotide primers 5'-GCGAA TTCTT CTCCC CTGGC TCTTCAG-3' ( $\alpha 6$  cDNA nucleotides 11–29), 5'-GCGGT ACCAC GGAGT GTAAA TCTGGAT-3' (antisense nucleotides 733–752), 5'-GCGGA TCCGA AGATGGGCTA TTTCATG-3' (nucleotides 714–732) and 5'-GCGGT ACCTC CAGCT TTTCC TGGCTGC-3' (antisense nucleotides 1,366–1,384). For PCR, 20 pmol primers were used in 50  $\mu$ l reaction volume and 35 cycles (94 °C, 45 s; 55 °C, 30 s; 72 °C, 120 s) were run in a programmable thermoblock (Perkin-Elmer). Amplified DNA fragments were gel-excised and cloned into M13 vectors<sup>19</sup> for sequence analysis<sup>10</sup>.

affected by the point mutation. Figure 2 shows that  $\alpha 6$  messenger RNA levels were comparable in ANT and control animals: and hence the differential binding of [<sup>3</sup>H]Ro15-4513 in the presence of increasing concentrations of diazepam in cerebellar sections of control and ANT rats does not reflect disparate cerebellar expression of  $\alpha 6$  subunit-containing GABA<sub>A</sub> receptors. This is in agreement with earlier work using photoaffinity labelling of these receptors with [<sup>3</sup>H]Ro15-4513 and gel analysis of labelled proteins<sup>3</sup>.

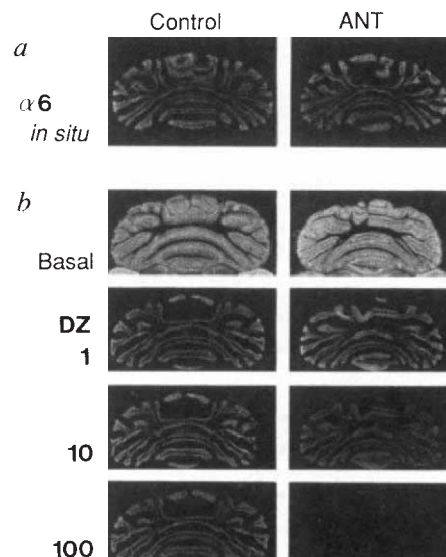
To demonstrate that the mutationally altered  $\alpha 6$  subunit imparts properties on GABA<sub>A</sub> receptors that are typical of the ANT rat cerebellar receptor,  $\alpha 6(Q100)\beta 2\gamma 2$  and  $\alpha 6\beta 2\gamma 2$  receptors were transiently expressed in human embryonic kidney (HEK) 293 cells and analysed by ligand binding and patch-clamp recording (Fig. 3). In the absence of diazepam, [<sup>3</sup>H]Ro15-4513 binding gave dissociation constant ( $K_D$ ) values of  $1.7 \pm 0.3$  nM and  $11.6 \pm 1.1$  nM (mean  $\pm$  s.e.m.;  $n = 3$ ) and maximal density of binding sites ( $B_{max}$ ) of  $3.1 \pm 0.1$  pmol per mg protein and  $5.6 \pm 0.3$  pmol per mg protein (mean  $\pm$  s.e.m.;  $n = 3$ ) to mutant and wild-type receptors. Similar differences in  $K_D$  values have previously been determined for cerebellar membranes of

ANT rats having negligible diazepam-insensitive [<sup>3</sup>H]Ro15-4513 binding, and of control rats<sup>3</sup>. Diazepam displaced [<sup>3</sup>H]Ro15-4513 binding to the mutant receptor with an inhibitory constant ( $K_i$ ) of  $1.3 \pm 0.1$   $\mu$ M ( $n = 4$ ), a value essentially identical to those ( $1.3$  to  $1.9$   $\mu$ M) determined from ANT cerebellar membranes<sup>3,13</sup>. Hence the recombinant GABA<sub>A</sub> receptor that incorporates the point mutation in  $\alpha 6$  reproduces ligand binding properties of the cerebellar ANT receptor.

Patch-clamp recording in the whole-cell mode<sup>14</sup> done on cells expressing  $\alpha 6(Q100)\beta 2\gamma 2$  or  $\alpha 6\beta 2\gamma 2$  receptors revealed that the mutant channels are also physiologically sensitive to benzodiazepine agonists, whereas channels containing the wild-type  $\alpha 6$  subunit are not (Fig. 3). Inward currents activated by GABA (100 nM) and recorded at  $-60$  mV were similar for both receptor channels (mutant,  $66 \pm 39$  pA, mean  $\pm$  s.e.m.,  $n = 6$ ; wild-type,  $65 \pm 37$  pA,  $n = 6$ ). This GABA concentration was well below the half-maximal effective concentration ( $EC_{50}$ ) value of  $\sim 300$  nM determined for  $\alpha 6\beta 2\gamma 2$  and  $\alpha 6(Q100)\beta 2\gamma 2$  channels (not shown). In mutant channels, currents activated by GABA (100 nM) were enhanced by  $48 \pm 9\%$  ( $n = 7$ ) in the presence of 10  $\mu$ M diazepam. Potentiation by diazepam was reversibly

FIG. 2 Comparable  $\alpha 6$  subunit mRNA expression by *in situ* hybridization (a) and differential displacement of [<sup>3</sup>H]Ro15-4513 binding by diazepam (b) in horizontal cerebellar sections from ANT and control rat brains. Grain accumulation on film was photographed as positive image.

**METHODS.** Frozen sections (14- $\mu$ m) cut from six ANT and six control brains were thaw-mounted onto gelatin-chrome alum-coated glass slides. Sections were preincubated for 15 min at 0 °C in 50 mM Tris-citrate (pH 7.3) supplemented with 120 mM NaCl, and incubated for 60 min in the same conditions with 6 nM [<sup>3</sup>H]Ro15-4513 (27 Ci mmol<sup>-1</sup>, New England Nuclear) in the presence of diazepam (DZ: 1, 10 or 100  $\mu$ M). Sections were washed twice for 30 s in the same buffer, dipped in distilled water, air-dried and exposed to Hyperfilm-<sup>3</sup>H (Amersham) for four weeks. Flumazenil (10  $\mu$ M) abolished all silver-grain accumulation to general background levels (not shown). For *in situ* hybridization<sup>21</sup>, a <sup>35</sup>S-labelled antisense oligonucleotide to  $\alpha 6$  cDNA nucleotides 1,081–1,125 (ref. 6) was used as probe. Parallel sections hybridized in the presence of 100-fold excess of unlabelled oligonucleotide showed background labelling (not shown).



blocked by Ro15-1788 (10  $\mu$ M;  $n = 5$ ), an antagonist at central benzodiazepine sites. In contrast to the mutant channel, and consistent with our previous finding<sup>15</sup>, diazepam (10  $\mu$ M) failed to potentiate responses to GABA (100 nM) in 10 cells expressing wild-type  $\alpha 6\beta 2\gamma 2$  receptors.

We propose that the potentiation of GABA<sub>A</sub> currents by benzodiazepine agonists in the mutant cerebellar  $\alpha 6$  receptor underlies the enhanced postural reflex sensitivity to such compounds in ANT rats. This is the first indication that a behavioural consequence (that is, impaired cerebellar motor control) may arise from a naturally occurring point mutation in a GABA<sub>A</sub> receptor subunit. The altered properties of the GABA<sub>A</sub> receptor incorporating the single amino-acid substitution emphasizes the importance of the residue in position 100 of the  $\alpha$ -subunits<sup>7</sup>. The simplest explanation for this may be that the arginine residue with its bulky side chain imposes a steric hindrance on access of benzodiazepine agonists to the allosteric modulatory site. This hindrance seems to be removed when histidine (in most  $\alpha$ -subunits) or glutamine (ANT  $\alpha 6$ ) occupy the critical position and hence, GABA<sub>A</sub> currents can now be potentiated by the appropriate compounds. Our results are compatible with the notion that sensitivity to alcohol and benzodiazepines may be governed at least partly by similar mechanisms, with a strong involvement of central GABA<sub>A</sub> receptors<sup>16</sup>. However, the  $\alpha 6(Q100)$  mutation does not generally underlie enhanced sensitivity to sedative drugs because several other rodent lines selected for increased alcohol sensitivity do not carry it, as judged from ligand-binding properties<sup>13</sup>. Moreover, in preliminary experiments, neither  $\alpha 6\beta 2\gamma 2$  nor  $\alpha 6(Q100)\beta 2\gamma 2$  receptors were modulated by ethanol.

A rat line expressing a mutant GABA<sub>A</sub> receptor only in cerebellar granule cells, whose drug-potentiated activation *in vivo* can be measured in terms of altered postural reflex behaviour may yield answers to unresolved issues. For example, we can now address the specific role of the  $\alpha 6$  subunit containing GABA<sub>A</sub> receptor in cerebellar circuits controlling movement. This might provide a rationale for the many GABA<sub>A</sub> receptor

TABLE 1 Analysis of the  $\alpha 6$  subunit gene in ANT and control rats

| Rat number and line | Diazepam-insensitive binding of cerebellar [ <sup>3</sup> H]Ro15-4513 (fmol per mg protein) | Hybridization to |             | Genomic $\alpha 6$ codon 100 |
|---------------------|---------------------------------------------------------------------------------------------|------------------|-------------|------------------------------|
|                     |                                                                                             | Q probe (%)      | R probe (%) |                              |
| 1. ANT              | 0                                                                                           | 100              | 0           | CAA (Q)                      |
| 2. Control          | 411                                                                                         | 0                | 100         | CGA (R)                      |
| 3. ANT              | 0                                                                                           | 100              | 0           | CAA (Q)                      |
| 4. Control          | 362                                                                                         | 0                | 100         | CGA (R)                      |
| 5. ANT              | 182                                                                                         | 51               | 49          | CAA, CGA                     |

ANT and control rats from the F<sub>40</sub> generation where pharmacologically characterized as detailed in ref. 3. Briefly the level of diazepam-insensitive [<sup>3</sup>H]Ro15-4513 binding was determined in triplicate for each animal in cerebellar samples, using 10 nM of the radioligand in the presence of 200  $\mu$ M diazepam. Genomic DNA isolated from the livers of these rats was used for PCR-mediated amplification<sup>9</sup> of  $\alpha 6$  subunit gene sequences with oligonucleotide primers 5'-GCGAA TTCGG ACTGA TGAGA GGCTGAAG-3' (nucleotides 260–279 and containing an *EcoRI* site for cloning) and 5'-GCGGT ACCAT GGTGT TAGAG GATCGTC-3' (antisense nucleotides 425–444 and containing a *KpnI* site). PCR was done with 200 ng genomic DNA and 20 pmol of each primer in a reaction volume of 100  $\mu$ l (premelting at 94 °C for 5 min, followed by 30 cycles of 94 °C, 20s; 55 °C, 30 s; 72 °C, 60s). Amplified DNA fragments were cloned into *EcoRI*-*KpnI*-digested M13mp18 replicative form DNA<sup>20</sup> and recombinant phage were filter-lifted. Filter sets were hybridized with <sup>32</sup>P-labelled antisense oligonucleotides specific for the wild-type sequence (R probe: 5'-CCCAT TTCGG AAAAA TGTGT-3'), and the ANT point mutation (Q probe: 5'-CCCAT TTTGG AAAAA TGTGT-3'), and as a control for recombinant plaques, with a radiolabelled  $\alpha 6$  cDNA probe (*AccI* fragment,  $\alpha 6$  nucleotides 129–695 containing most of the  $\alpha 6$  extracellular region coding sequence). Hybridization was overnight in 30% formamide, 5  $\times$  SSC (1  $\times$  SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 32 °C (oligonucleotides), or in 50% formamide, 5  $\times$  SSC at 42 °C (*AccI* fragment); excess radioactivity was removed by two washes in 0.4  $\times$  SSC for 15 s at 50 °C (oligonucleotides) or for 5 min at 60 °C (*AccI* fragment). Filters were exposed to Kodak XAR X-ray film. Numbers of  $\alpha 6$  glutamine(100) and arginine(100) forms were evaluated by counting the respective signal-carrying plaques<sup>12</sup>. The total number of recombinant plaques analysed exceeded 300 for each genomic sample.

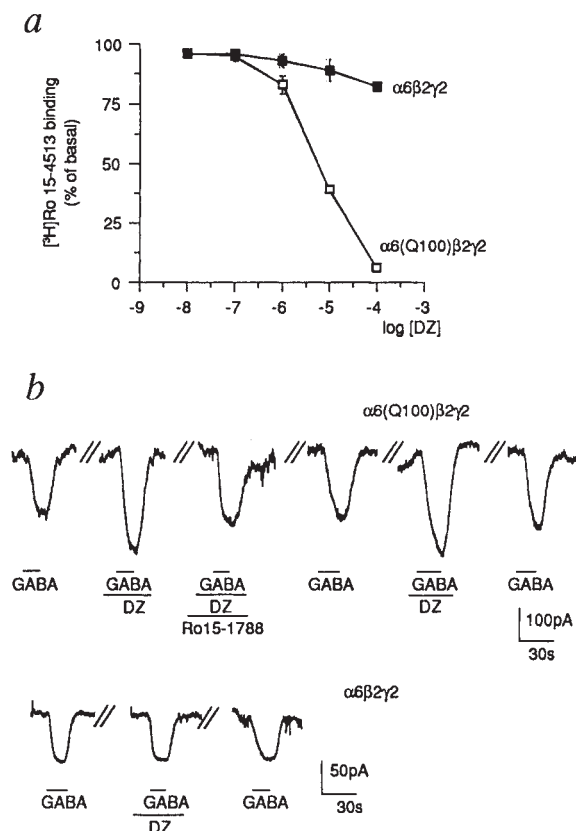


FIG. 3 Displacement curves by diazepam of [<sup>3</sup>H]Ro15-4513 binding (a) and potentiation by diazepam of GABA-activated currents (b) in  $\alpha 6(Q100)\beta 2\gamma 2$  and  $\alpha 6\beta 2\gamma 2$  receptor channels.

METHODS. The  $\alpha 6(Q100)$  mutation was introduced into a cytomegalovirus promoter-based expression vector<sup>22</sup> for  $\alpha 6$  by exchanging a *BspEI*-*PvuMI* fragment (nucleotides 340–591) between the wild-type<sup>6</sup> and ANT  $\alpha 6$  cDNAs. HEK 293 cells (ATCC CRL 1573) were transfected<sup>23</sup> with vectors for the rat GABA<sub>A</sub> receptor subunit  $\alpha 6$  (wild-type or mutant),  $\beta 2$  and  $\gamma 2$  (ref. 15). Cultures were re-fed after 20 h, and 48 h post-transfection, cells were collected in phosphate-buffered saline. Cell membranes were washed twice in ice-cold 50 mM Tris-citrate buffer (pH 7.3) by resuspension and centrifugation. The binding of 6 nM [<sup>3</sup>H]Ro15-4513 in the presence of various concentrations of diazepam and that of 1–48 nM [<sup>3</sup>H]Ro15-4513 in the absence of diazepam (not shown) was determined in duplicate samples during 60-min incubations at 0 °C in Tris-citrate buffer<sup>24</sup>. Flumazenil at 10  $\mu$ M defined nonspecific binding which was  $4.0 \pm 0.5\%$  (mean  $\pm$  s.e.m.,  $n = 8$ ) of total binding at 6 nM [<sup>3</sup>H]Ro15-4513. Values are means  $\pm$  s.e.m. from 4 experiments. The  $K_D$  and  $B_{max}$  values for the saturation isotherms and the  $K_i$  values for the displacement of [<sup>3</sup>H]Ro15-4513 binding by diazepam were estimated using the Inplot program (Graph Pad Software, San Diego, CA). Transfected cells growing on fibronectin-coated glass coverslips were patch-clamped in the whole-cell mode<sup>14</sup> at 25 °C. The pipette solution contained in mM: KCl 130, MgCl<sub>2</sub> 4, CaCl<sub>2</sub> 1, EGTA 10, HEPES 10. Cells were bath-perfused with HEPES-buffered (pH 7.2) salt solution in mM: NaCl 129, KCl 5.4, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1, D-glucose 5. All recordings were made with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, FRG) with the membrane potential clamped at -60 mV. Representative GABA-induced current traces from one  $\alpha 6(Q100)\beta 2\gamma 2$  and one  $\alpha 6\beta 2\gamma 2$  receptor-expressing cell are depicted. GABA was at 100 nM, diazepam (DZ) and flumazenil (Ro15-1788) at 10  $\mu$ M. Successive bath applications indicated by bars below current traces were separated by 5-min intervals to minimize desensitization.



variants used by the CNS. As ANT rats have a diminished behavioural reaction to stress, even in the absence of pharmacological treatment<sup>17</sup>, this rat line could provide further clues on the existence in brain of endogenous ligands that modulate GABA<sub>A</sub> currents by means of the benzodiazepine site<sup>18,19</sup>. □

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1. Eriksson, K. & Rusi, M. in *Development of Animal Models as Pharmacogenetic Tools* (eds McClearn, G. E., Deitrich, G. E. & Erwin, G.) 87–117 (US Government Printing Office, Washington DC 1981).
2. Hellevo, K., Kiiianmaa, K. & Korpi, E. R. *Pharmac. biochem. Behav.* **34**, 399–404 (1989).
3. Uusi-Oukari, M. & Korpi, E. R. *J. Neurochem.* **54**, 1980–1987 (1990).
4. Sieghart, W., Eichinger, A., Richards, J. G. & Möhler, H. *J. Neurochem.* **48**, 46–52 (1987).
5. Malmiemi, O. & Korpi, E. R. *Eur. J. Pharmac.* **169**, 53–60 (1989).
6. Lüddens, H. et al. *Nature* **346**, 648–651 (1990).
7. Wieland, H. A., Lüddens, H. & Seeburg, P. H. *J. Biol. Chem.* **267**, 1426–1429 (1992).
8. Wisden, W. & Seeburg, P. H. *Curr. Opin. Neurobiol.* **2**, 263–269 (1992).
9. Mullis, K. B. & Faloona, F. A. *Meth. Enzym.* **155**, 335–356 (1987).
10. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
11. Powell, L. M. et al. *Cell* **50**, 831–840 (1987).
12. Sommer, B., Köhler, M., Sprengel, R. & Seeburg, P. H. *Cell* **67**, 11–19 (1991).
13. Uusi-Oukari, M. & Korpi, E. R. *Alc. Clin. exp. Res.* **15**, 241–246 (1991).
14. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch.* **391**, 85–100 (1981).
15. Kleingor, C., Ewert, M., von Blankenfeld, G., Seeburg, P. H. & Kettenmann, H. *Neurosci. Lett.* **130**, 169–172 (1991).
16. Harris, R. A. & Allan, A. *FASEB J.* **3**, 1689–1695 (1989).
17. Tuominen, K. & Korpi, E. R. *Pharmac. Biochem. Behav.* **40**, 409–415 (1991).
18. Sangameswaran, L. & DeBlas, A. L. *Proc. natn. Acad. Sci. U.S.A.* **82**, 5560–5564 (1985).
19. Rothstein, J. D. et al. *J. Neurochem.* **58**, 2102–2115 (1992).
20. Vieira, J. & Messing, J. *Meth. Enzym.* **153**, 3–11 (1987).
21. Wisden, W., Laurie, D. J., Monyer, H. & Seeburg, P. H. *J. Neurosci.* **12**, 1040–1062 (1992).
22. Schall, T. J. et al. *Cell* **61**, 361–370 (1990).
23. Chen, C. & Okayama, H. *Molec. cell. Biol.* **7**, 2745–2752 (1987).
24. Pritchett, D. B. et al. *Nature* **338**, 582–585 (1989).

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## Phagocytic processing of bacterial antigens for class I MHC presentation to T cells

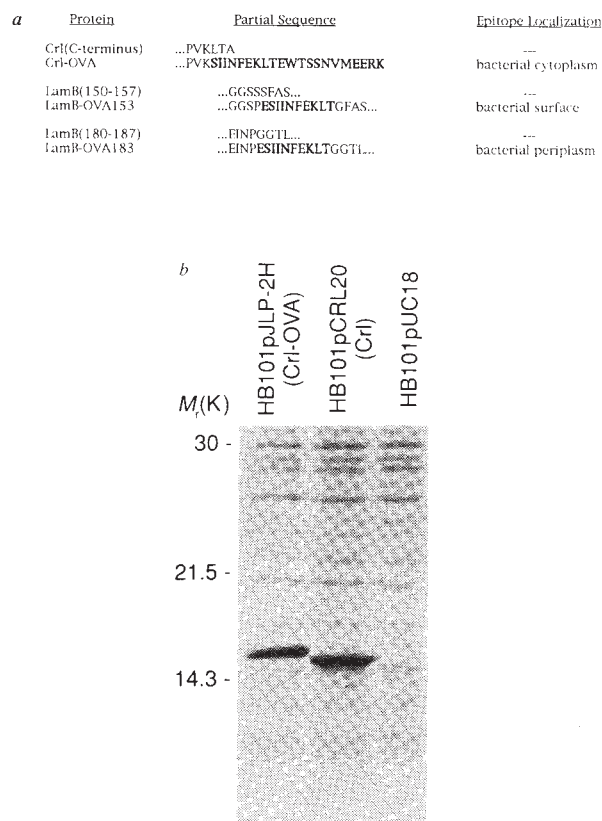
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**CLASS I major histocompatibility complex (MHC) molecules present antigens that are produced within the presenting cell or penetrate from the vacuolar system into the cytosol for processing. Most studies of exogenous antigen processing have used soluble antigens, which are not efficiently presented by class I MHC molecules<sup>1</sup> and do not elicit CD8 T-cell responses *in vivo*. But particulate antigen preparations with no known mechanism for cytosolic penetration can also elicit CD8 T-cell responses *in vivo*<sup>2–7</sup>. We report here that phagocytosis of bacteria with no mechanism for cytosolic penetration also results in presentation of bacterial antigens by class I MHC molecules. Moreover, this mechanism is resistant to cycloheximide and Brefeldin A, which block the classical class I processing pathway. These results suggest a novel vacuolar class I processing pathway for exogenous phagocytic antigens.**

We used the 257–264 epitope of ovalbumin (OVA) that binds the H-2K<sup>b</sup> murine class I MHC molecule<sup>8</sup>. This epitope was expressed within fusion proteins in *Escherichia coli* or *Salmonella typhimurium*. DNA encoding residues 257–277 of OVA was

inserted into the carboxy-terminal region of *crl*, an *E. coli* gene encoding a bacterial cytoplasmic protein that regulates curli surface organelle expression<sup>9,10</sup>. Crl-OVA was constitutively expressed (100 ng per 10<sup>6</sup> bacteria) in the cytoplasm of HB101/pCrl-OVA (Fig. 1). The OVA(256–265) epitope was



**FIG. 1** a, Crl-OVA, LamB-OVA153, and LamB-OVA183 fusion proteins (OVA sequence in bold face). b, SDS-PAGE analysis of 7 × 10<sup>6</sup> boiled whole *E. coli* HB101 expressing Crl-OVA or Crl (stained with Coomassie blue). 10<sup>6</sup> HB101/pCrl-OVA bacteria contained about 0.1–0.2 μg Crl-OVA based on densitometric comparison with HB101 expressing the previously defined fusion protein, Crl-HEL<sup>22</sup>.

**METHODS.** For Crl-OVA, oligonucleotide primers incorporating *EcoRV* restriction sites were used to amplify the segment of the OVA gene encoding amino-acids 257–277 (ref. 23) using polymerase chain reaction (PCR). The DNA was digested with *EcoRV*, ligated into *HpaI*-digested pCRL20 DNA (refs 9, 22) and transformed into *E. coli* HB101. Crl-OVA retained the wild-type function of Crl in curli formation as assessed by fibronectin binding<sup>9</sup>. The entire *lamB* gene from *E. coli* YME1 was amplified by PCR using oligonucleotide primers that created an *EcoRI* site at the 5' end, a *BamHI* site at the 3' end, and a *SmaI* site restricting either between codons 153 and 154 or between codons 183 and 184. DNA encoding OVA(256–265) was synthesized as complementary overlapping 30-mer oligonucleotides that were phosphorylated, annealed, and ligated into *SmaI*-digested *lamB* DNA. The genes were then subcloned into the *EcoRI*–*BamHI* sites of pUHE21-2 (ref. 24) (modified by insertion of *lacI*<sup>9</sup>) and expressed in the *E. coli* *lamB* deletion strain RAM191 (ref. 25). Maltoporin function and outer membrane location of LamB-OVA153 and LamB-OVA183 were confirmed by growth in minimal medium containing maltotetraose, maltopentaose or maltohexaose<sup>25</sup>, with doubling times of <4 h for RAM191/LamB-OVA153, RAM191/LamB-OVA183 and the wild type *lamB*<sup>+</sup> parent MC4100, and >43 h for RAM191/pUHE21-2. The surface or periplasmic location of the OVA(256–265) epitope in LamB-OVA153 or LamB-OVA183, respectively, was confirmed by bacteriophage lambda sensitivity<sup>11,26</sup>. RAM191/pLamB-OVA183 was sensitive to wild-type phage; RAM191/pLamB-OVA153 was not, indicating altered surface phage-binding determinants in LamB-OVA153. Whole-cell ELISA<sup>41</sup> using anti-OVA serum also demonstrated much greater reactivity of RAM191/pLamB-OVA153 compared with both RAM191/pLamB-OVA183 and RAM191/pUHE21-2.

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inserted at sites within LamB to localize it to either a periplasmic (LamB-OVA183) or an extracellular (LamB-OVA153) domain of this outer membrane protein<sup>11</sup>, and expressed at various levels after induction. The fusion proteins retained the functions of wild-type Crl (curli regulation) or LamB (maltodextrin transport).

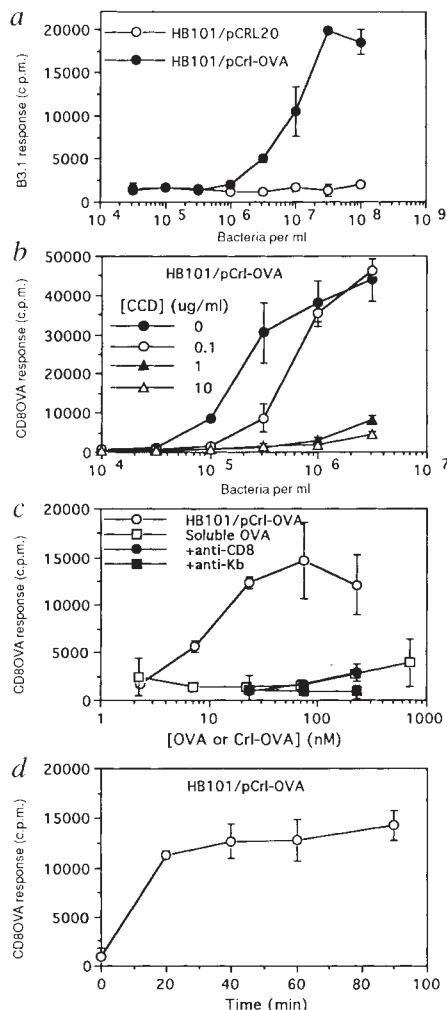


FIG. 2 Processing of bacterial Crl-OVA for class I MHC presentation. *a*, Response of B3.1 T cells to HB101/pCrl-OVA (expressing Crl-OVA) and to HB101/pCrl20 (expressing wild-type Crl). *b*, Response of CD8OVA T cells to HB101/pCrl-OVA with cytochalasin D present at 0–10  $\mu\text{g ml}^{-1}$ . Cytochalasin D did not block the presentation of soluble OVA(257–264) peptide (added with cytochalasin D before macrophage fixation, as above). *c*, Presentation of bacterial Crl-OVA was blocked by supernatants containing 3.155 anti-CD8 (ref. 27) or Y-3 and AF6.88.5.3 anti-K<sup>b</sup> monoclonal antibodies (ATCC, Rockville). No presentation was observed with equivalent titres of soluble crude Crl-OVA prepared by sonication and centrifugation of HB101/pCrl-OVA, although higher levels of soluble OVA (such as 1  $\text{mg ml}^{-1}$ ) did elicit presentation (data not shown). *d*, Kinetics of HB101/pCrl-OVA processing for K<sup>b</sup> presentation.

**METHODS.** Peritoneal macrophages were collected from C57BL/6 mice 5–14 days after intraperitoneal infection with  $10^4$  *Listeria monocytogenes*. Recombinant *E. coli* were added to adherent macrophages in 100  $\mu\text{l}$ , centrifuged at 2,500g for 5 min and incubated at 37 °C for 60–120 min (shorter in *d*). The macrophages were washed and fixed in 1% paraformaldehyde in PBS. T hybridoma cells specific for OVA(257–264)-K<sup>b</sup> were then added for 24 h; their interleukin-2 (IL-2) secretion was quantified by  $^3\text{H}$ -thymidine incorporation by IL-2-dependent CTLL cells. Two sets of T hybridomas were used: B3.1 (ref. 28) and CD8OVA (the latter were obtained by immunizing with liposome-encapsulated OVA<sup>5</sup>, expanding immune splenocytes *in vitro* with irradiated E.G7-OVA<sup>29</sup> and fusing them with T-cell receptor-deficient CD8-transfected BW5147 cells<sup>30</sup>). Error bars in all figures represent one standard deviation of triplicate cultures.

Elicited murine peritoneal macrophages were incubated with bacteria expressing recombinant antigen and then fixed, washed and incubated with CD8-positive T-cell hybridomas specific for OVA(257–264)-K<sup>b</sup> (B3.1 or CD8OVA cells). Bacterial Crl-OVA was efficiently processed and specifically presented to these T cells, whereas bacterial Crl was not recognized (Fig. 2). About  $3 \times 10^4$  bacteria added to  $10^5$  macrophages in 0.1 ml cultures elicited class I-restricted T-cell responses. Presentation was detected within 20 min (Fig. 2d). Soluble OVA or Crl-OVA was presented less efficiently by K<sup>b</sup> (Fig. 2c).

We excluded the possibility that spontaneous bacterial release of Crl-OVA, independent of the phagocytic pathway, was generating a pool of extracellular peptides that could bind directly to class I MHC molecules on the plasma membrane of macrophages, because presentation was inhibited by the addition of cytochalasin D to block phagocytic uptake (Figs 2b and 3a). Fixation of the macrophages before the introduction of bacteria also blocked processing for class I MHC presentation (Fig. 3a), whereas prefixed cells could still present peptide antigen (Fig. 3f, and data not shown). EL4 cells (non-phagocytic) did not process and present bacterial Crl-OVA, although they did present soluble OVA(257–264) peptide. Thus, phagocytic processing was essential to this pathway for class I presentation.

Conventional class I processing depends on nascent class I MHC molecules and is blocked by cycloheximide and Brefeldin A. Accordingly, when soluble OVA was introduced into the macrophage cytosol by electroporation<sup>12</sup>, Brefeldin A inhibited its presentation by K<sup>b</sup> (Fig. 3c). But the processing of bacterial Crl-OVA was unaffected by these inhibitors (Fig. 3b). Thus phagocytic class I processing was not dependent on nascent class I MHC molecules. Moreover, viable MHC-disparate macrophages released bacterial peptides that were presented by adjacent, fixed MHC-matched macrophages (Fig. 3f). This indicated that the bacterial antigens were first processed in phagocytic compartments, and processed peptides were then regurgitated to bind to surface class I MHC molecules.

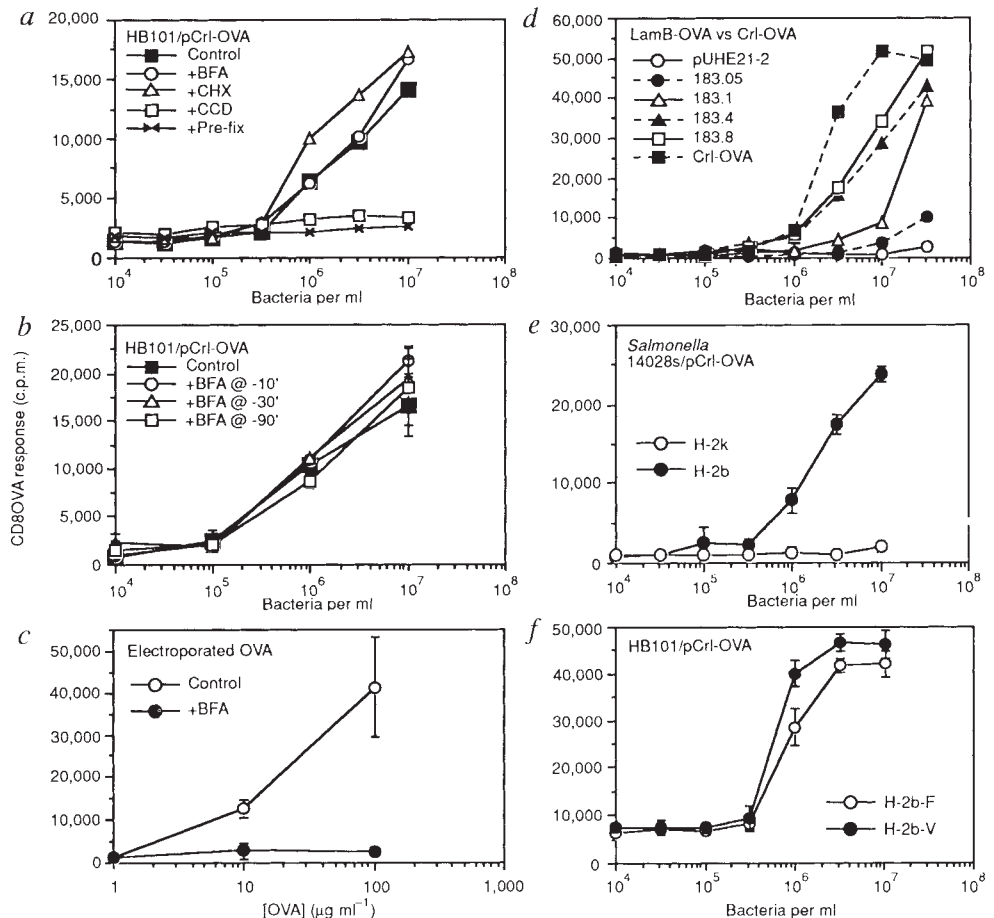
Bacterial LamB-OVA153 and LamB-OVA183 were also processed for presentation by K<sup>b</sup> (Fig. 3d), generalizing class I processing to include internal epitopes from bacterial membrane proteins situated either on the cell surface or in the periplasm. Bacteria with varying levels of LamB-OVA or Crl-OVA elicited presentation with corresponding variation in efficiency. Moreover, Crl-OVA expressed in *S. typhimurium* was similarly processed (Fig. 3e). Thus, the same results were obtained with several soluble or transmembrane fusion proteins; with expression of the antigenic epitope in the cytoplasm, periplasm or cell surface; with expression at varying levels; and with expression in two different intravacuolar bacterial species, including a pathogenic strain of *Salmonella*.

Electron microscopy showed that *E. coli* remained confined to vacuolar compartments without egress to the cytosol for at least 60 min after phagocytosis. Phagosomes containing bacteria fused extensively with lysosomes within 20 min (Fig. 4). Intracellular *Salmonella* have also been found to remain within vacuolar compartments<sup>13</sup>.

CD8 T-cell responses are elicited *in vivo* by microbes without defined egress from the vacuolar system into the cytosol<sup>14–17</sup>, but the cellular basis for these responses has been unclear. We now demonstrate a phagocytic antigen processing pathway for presentation of bacterial antigens by class I MHC molecules. This pathway seems most efficient for particulate antigen, although higher doses of soluble antigen can elicit class I MHC presentation<sup>18,19</sup>. A similar pathway may be followed by several particulate antigen formulations which do not actively penetrate into the cytosol, including acid-resistant liposomes<sup>5,6</sup>, mammalian cells<sup>2–4,7</sup>, and ovalbumin conjugated to latex microspheres (C.V.H., unpublished observations). All of these formulations elicit CD8 T-cell responses *in vivo*, in contrast to soluble antigens<sup>5</sup>.



FIG. 3 a, HB101/pCrl-OVA processing with Brefeldin A (BFA,  $0.5 \mu\text{g ml}^{-1}$ ), cycloheximide (CHX,  $10 \mu\text{g ml}^{-1}$ ), and cytochalasin D (CCD,  $10 \mu\text{g ml}^{-1}$ ) added 10 min before HB101/pCrl-OVA. 'Pre-fix' indicates macrophages that were fixed before the addition of bacteria. b, Brefeldin A ( $0.5 \mu\text{g ml}^{-1}$ ) was added 10, 30 or 90 min before HB101/pCrl-OVA. c, Soluble OVA was introduced into the macrophage cytosol by electroporation in the presence of OVA at the indicated concentrations. The cells were resuspended with or without Brefeldin A at  $0.5 \mu\text{g ml}^{-1}$  for 2 h with rotation<sup>31</sup> before fixation. d, Crl-OVA and LamB-OVA presentation with varying antigen expression. *E. coli* strain RAM191/pLamB-OVA183 was cultured overnight in LB (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) at  $37^\circ\text{C}$  and then diluted in LB to an optical density at 600 nm ( $\text{OD}_{600}$ ) = 0.1. After growth to  $\text{OD}_{600}$  = 0.8, isopropyl- $\beta$ -D-thiogalactoside (IPTG) was added at 0.05–0.8 mM for 45 min to induce expression of LamB-OVA183 (periplasmic OVA epitope). Protein expression was assessed by ELISA<sup>22</sup> using anti-OVA to detect Crl-OVA and LamB-OVA. The following relative levels of OVA epitope were detected: Crl-OVA, relative level 1; LamB-OVA183.8, relative level 0.3; LamB-OVA183.4, relative level 0.24; LamB-OVA183.1, relative level 0.09; LamB-OVA183.05, relative level 0.05; RAM191/pUHE21-2, relative level 0 (vector control). e, Processing of *S. typhimurium* strain 14028s expressing Crl-OVA by C57BL/6 macrophages (H-2<sup>b</sup>) or CBA/J macrophages (H-2<sup>k</sup>). f, Adherent H-2<sup>k</sup> or H-2<sup>b</sup> macrophages ( $5 \times 10^4$  per well) were fixed (F) and washed, and viable (V) macrophages of the other haplotype were then added. After incubation with HB101/pCrl-OVA the cells were again fixed, and



presentation by the H-2<sup>b</sup> macrophages was determined. No CD8OVA response was observed with viable H-2<sup>k</sup> macrophages alone processing HB101/pCrl-OVA (not shown).

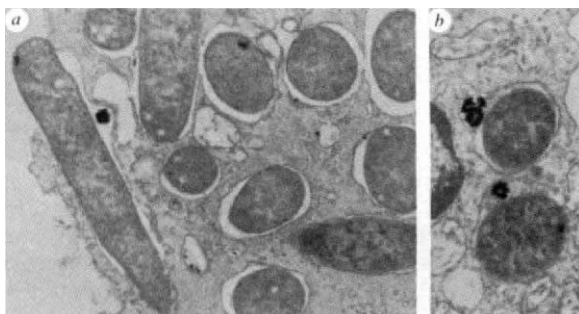


FIG. 4 Electron microscopy of macrophages after bacterial phagocytosis. a, After phagocytosis HB101/pCrl-OVA was confined to vacuolar compartments without egress into the cytosol. Lysosomes were labelled by uptake of colloidal gold-BSA with a 12 h chase. Note delivery of colloidal gold lysosomal marker into the nascent phagosome without complete internalization, suggesting a possible pathway for peptide regurgitation, although extensive bacterial degradation to release Crl-OVA may require internal phagolysosomal processing. Magnification  $\times 30,500$ . b, Extensive fusion of lysosomes with phagosomes occurs within 20 min. Note colocalization of colloidal gold and bacteria. Magnification  $\times 38,400$ .

**METHODS.** To label lysosomes, adherent peritoneal macrophages were incubated with 10 nm colloidal gold-BSA for 5 h, washed and incubated for 12 h in normal medium plus indomethacin ( $10^{-6}$  M) and recombinant murine interferon- $\gamma$  ( $30 \text{ ng ml}^{-1}$ ). HB101/pCrl-OVA were added ( $10^8 \text{ ml}^{-1}$ ) for 20 min. The cells were then fixed in 3% glutaraldehyde in cacodylate buffer, scraped loose, and processed for transmission electron microscopy (en block staining with uranyl acetate, embedding in Spurr's resin, and staining with lead citrate).

The mechanism of this alternative class I processing pathway appears to be distinct from active egress of bacteria into the cytosol, as with *Listeria monocytogenes*<sup>20</sup>, because *E. coli* and *S. typhimurium* both remain within vacuolar compartments. The rapid kinetics of this pathway are also consistent with processing within vesicular organelles. Moreover, the resistance to Brefeldin A and cycloheximide suggests that post-Golgi class I MHC molecules may be used, consistent with the observed regurgitation of processed peptides for presentation by surface class I molecules on fixed cells. Peptide regurgitation may be an important mechanism whereby non-phagocytic cells, such as dendritic cells, can acquire and present microbial peptides. In viable phagocytic cells class I MHC molecules may be loaded either on the cell surface (by peptide regurgitation) or within vacuolar compartments after reinternalization, using previously occupied class I MHC molecules (by peptide exchange)<sup>21</sup> or unoccupied class I MHC molecules. This alternative class I pathway may allow induction of CD8 T-cell responses to intracellular pathogens that remain within the vacuolar system. □

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- Braciale, T. J. *et al. Immun. Rev.* **98**, 95–114 (1987).
- Carbone, F. R. & Bevan, M. J. *J. exp. Med.* **171**, 377–387 (1990).
- Bevan, M. J. *J. exp. Med.* **143**, 1283–1288 (1976).
- Gooding, L. R. & Edwards, C. B. *J. Immun.* **124**, 1258–1262 (1980).
- Collins, D. S., Findlay, K. & Harding, C. V. *J. Immun.* **148**, 3336–3341 (1992).
- Reddy, R., Zhou, F., Nair, S., Huang, L. & Rouse, B. T. *J. Immun.* **148**, 1585–1589 (1992).
- Debrick, J. E., Campbell, P. A. & Staerz, U. D. *J. Immun.* **147**, 2846–2851 (1991).
- Falk, K., Rötzschke, O., Stevanovic, S., Jung, G. & Rammensee, H.-G. *Nature* **351**, 290–296 (1991).
- Olsen, A., Jonsson, A. & Normark, S. *Nature* **338**, 652–655 (1989).
- Arnqvist, A., Olsen, A., Pfeifer, J., Russell, D. & Normark, S. *Molec. Microbiol.* **6**, 2443–2452 (1992).

11. Charbit, A., Ronco, J., Michel, V., Werts, C. & Hofnung, M. *J. Bact.* **173**, 262–275 (1991).
12. Harding, C. V. *Eur. J. Immun.* **22**, 1865–1869 (1992).
13. Finlay, B. B. & Falkow, S. *Molec. Microbiol.* **3**, 1833–1841 (1989).
14. Farrell, J. P., Muller, I. & Louis, J. A. *J. Immun.* **142**, 2052–2056 (1989).
15. Flynn, J. L. et al. *Molec. Microbiol.* **4**, 2111–2118 (1990).
16. Aggarwal, A. et al. *J. exp. Med.* **172**, 1083–1090 (1990).
17. Gao, X.-M. et al. *Infect. Immun.* **60**, 3780–3789 (1992).
18. Rock, K. L., Gamble, S. & Rothstein, L. *Science* **249**, 918–921 (1990).
19. Grant, E. P. & Rock, K. L. *J. Immun.* **148**, 13–18 (1992).
20. Brunt, L. M., Portnoy, D. A. & Unanue, E. R. *J. Immun.* **145**, 3540–3546 (1990).
21. Smith, J. D., Lie, W.-R., Gorka, J., Myers, N. B. & Hansen, T. H. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7767–7771 (1992).
22. Pfeifer, J. D., Wick, M. J., Russell, D. G., Normark, S. J. & Harding, C. V. *J. Immun.* **149**, 2576–2584 (1992).
23. McReynolds, L. et al. *Nature* **273**, 723–728 (1978).
24. Lanzer, M. & Bujard, H. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8973–8977 (1988).
25. Misra, R. & Benson, S. A. *J. Bact.* **170**, 528–533 (1988).
26. Charbit, A., Boulain, J. C., Ryter, A. & Hofnung, M. *EMBO J.* **5**, 3029–3037 (1986).
27. Sarmiento, M. et al. *Immun. Rev.* **68**, 133–169 (1982).
28. Harding, C. V., Collins, D. S., Kanagawa, O. & Unanue, E. R. *J. Immun.* **147**, 2860–2863 (1991).
29. Carbone, F. R. & Bevan, M. J. *J. exp. Med.* **169**, 603–612 (1989).
30. Burger, H.-G., White, J., Weltzien, H.-U., Marrack, P. & Kappler, J. W. *J. exp. Med.* **170**, 1887–1904 (1989).
31. Harding, C. V. & Unanue, E. R. *J. Immun.* **147**, 767–773 (1991).

## Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1(7-37)

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**NON-INSULIN-DEPENDENT diabetes mellitus (NIDDM, type 2 diabetes) is a disorder of glucose homeostasis characterized by hyperglycaemia, peripheral insulin resistance, impaired hepatic glucose metabolism, and diminished glucose-dependent secretion of insulin from pancreatic  $\beta$ -cells<sup>1</sup>. Glucagon-like-peptide-1(7-37) (GLP-1)<sup>2</sup> is an intestinally derived hormone that may be useful for the treatment of NIDDM because it acts *in vivo* to increase the level of circulating insulin, and thus lower the concentration of blood glucose<sup>3,4</sup>. This therapeutic effect may result from the ability of GLP-1 to compensate for a defect in the glucose signalling pathway that regulates insulin secretion from  $\beta$ -cells. In support of this concept we report here that GLP-1 confers glucose sensitivity to glucose-resistant  $\beta$ -cells, a phenomenon we term glucose competence. Induction of glucose competence by GLP-1 results from its synergistic interaction with glucose to inhibit metabolically regulated potassium channels that are also targeted for inhibition by sulphonylurea drugs commonly used in the treatment of NIDDM<sup>5</sup>. Glucose competence allows membrane depolarization, the generation of action potentials, and  $\text{Ca}^{2+}$  influx, events that are known to trigger insulin secretion<sup>6,7</sup>.**

GLP-1 exerts diverse insulinotropic actions on  $\beta$ -cells that include stimulation of cyclic AMP formation<sup>8</sup>, insulin secretion<sup>9</sup>, insulin biosynthesis and proinsulin gene expression<sup>10</sup>. Because all insulinotropic actions of GLP-1 are dependent on simultaneous exposure of  $\beta$ -cells to glucose<sup>10,11</sup>, GLP-1 may act as a hormonal regulator, or modulator, of the  $\beta$ -cell glucose signalling system. To investigate this possibility, perforated-patch<sup>12–14</sup> and cell-attached-patch<sup>15</sup> recordings were obtained from solitary  $\beta$ -cells isolated from dispersed rat islets of Langerhans and maintained in short-term primary cell culture<sup>16</sup>. For unknown reasons, these single, isolated  $\beta$ -cells exhibit reduced sensitivity to glucose as measured by glucose-induced insulin secretion<sup>17</sup>, electrical activity<sup>18</sup> or calcium signalling<sup>19</sup>. When perforated-patch recordings were obtained from such cells under conditions

in which the bathing solution contained no glucose, the depolarizing responses to 10 mM glucose were often  $\leq 10$ –15 mV in amplitude, and glucose did not always initiate repetitive spiking activity as is frequently observed in intact islets<sup>6</sup> (Fig. 1a trace 1). In addition, in the absence of glucose very little change in membrane potential was recorded in response to 10 nM GLP-1 (Fig. 1a left of trace 2). In marked contrast, a 30–35 mV depolarization accompanied by repetitive spiking activity was often observed when glucose and GLP-1 were applied simultaneously (Fig. 1a right of trace 2). This synergistic interaction indicates that GLP-1 acts in a manner analogous to that of a modulatory transmitter. It enhances the responsiveness of  $\beta$ -cells to glucose, yet is without effect in the absence of glucose.

The interaction of GLP-1 and glucose is remarkable in that the timing of the application of the two substances need not be simultaneous. Glucose-insensitive  $\beta$ -cells were rendered glucose-competent (capable of responding to glucose) by pretreatment with GLP-1 (Fig. 1b trace 1). Conversely, GLP-1-insensitive cells were rendered GLP-1-sensitive by prior application of glucose (Fig. 1b trace 2). These 'priming' effects associated with GLP-1 or glucose pretreatment may reflect either the prolonged action of cytosolic second messengers and/or slow dissociation of GLP-1 from its receptor.

Synergism between glucose and GLP-1 was dose-dependent (effector concentration for half maximum response,  $\text{EC}_{50}$ , 1 nM), and exhibited pharmacological specificity because deletion of a single amino acid at the amino terminus to yield GLP-1(8-37)<sup>21</sup> abrogated activity (Fig. 1c, left). For these reasons, the action of GLP-1 is mediated by a receptor with pharmacological properties similar to that which mediates GLP-1-induced stimulation of insulin secretion from insulinoma cell lines<sup>10,22,23</sup>. That the action of GLP-1 is in fact a receptor-mediated process is supported by a recent report of the complementary DNA cloning of a  $\beta$ -cell GLP-1 receptor that binds GLP-1 (but not glucagon) with high affinity and which stimulates cAMP production<sup>24</sup>.

The induction of glucose competence by GLP-1 was specific for a distinct subpopulation of  $\beta$ -cells (Fig. 2a, b). When initially challenged with 10 mM glucose for 30 s, most cells exhibited either no response or a small ( $\leq 15$  mV) depolarizing response (Fig. 2a, cross-hatched bars). The glucose-sensitivity of individual  $\beta$ -cells therefore is diminished in comparison to  $\beta$ -cells of whole, intact islets. In contrast, when glucose-insensitive  $\beta$ -cells were subsequently challenged with a combined application of glucose and GLP-1, most cells were depolarized by  $\geq 30$  mV (Fig. 2a, cross-hatched bars). Scatter plot analysis revealed that the interaction between GLP-1 and glucose segregated among three distinct sets of observations (Fig. 2b, sets 1–3). Cells included in set 2 comprised 28% of all cells tested and were fully responsive to glucose (that is, constitutively glucose-competent). In contrast, cells included in sets 1 and 3 exhibited very little response when initially challenged with glucose, and these comprised 64% of all cells tested. When the cells that showed a poor initial response to glucose were subsequently challenged with glucose and GLP-1, introduction of glucose competence was observed in 40% of all cells tested, and it was this subpopulation of  $\beta$ -cells that comprised set 3. These findings are consistent with previous reports of microheterogeneity among  $\beta$ -cells<sup>16,17,19</sup>.

To begin to define the signalling system by which GLP-1 acts, the actions of membrane-permeant cAMP analogues were tested for their effects on the induction of glucose competence. As summarized in Table 1, in  $\beta$ -cells that initially exhibited a weak depolarizing response ( $< 15$  mV) to 10 mM glucose, the application of 10  $\mu\text{M}$  Rp-cAMPS, an antagonist of endogenous cAMP signalling pathways, inhibited the synergistic depolarizing interaction between glucose and GLP-1. In contrast, 10  $\mu\text{M}$  of the cAMP agonist Sp-cAMPS potentiated the depolarizing response to glucose even in the absence of GLP-1. These findings suggest

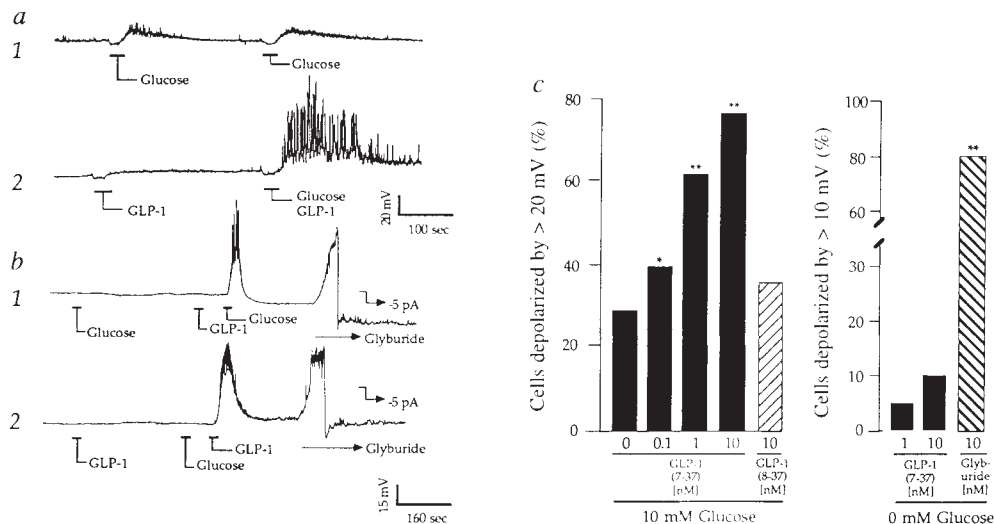
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FIG. 1 Synergism of glucose and GLP-1 to depolarize  $\beta$ -cells. **a**, Trace 1: membrane potential recordings from a  $\beta$ -cell exhibiting weak sensitivity to 10 mM glucose repeatedly applied for 30 s as indicated. Trace 2: recordings from the same cell during application of 10 nM GLP-1 (left) or combined application of 10 mM glucose and 10 nM GLP-1 (right). Initial resting potential ( $V_m$ ) = -62 mV. **b**, trace 1: a cell that initially failed to respond to 10 mM glucose, but which responded to 10 mM glucose after pretreatment with 10 nM GLP-1 ( $V_m$  = -66 mV). Trace 2: a cell that initially failed to respond to 10 nM GLP-1, but which responded to 10 nM GLP-1 after pretreatment with 10 mM glucose ( $V_m$  = -60 mV). The initial insensitivity of  $\beta$ -cells to glucose or GLP-1 did not result from their failure to express functional  $I_{KATP}$  channels because

application of 10 nM glyburide resulted in rapid membrane depolarization ( $42 \pm 11$  mV,  $\pm$ s.e.m.,  $n=10$  cells), as illustrated (**b**, traces 1, 2). **b**, -5 pA of hyperpolarizing pipette current was applied to repolarize the membrane after application of 10 nM glyburide. Repolarization confirms the integrity of the membrane/pipette seal. **c**, Cumulative dose-response analysis summarizing the interaction of glucose and GLP-1 to depolarize  $\beta$ -cells. The action of GLP-1(7-37) but not GLP-1(8-37) exhibited dose-dependence under conditions in which the test solution also contained 10 mM glucose (left). In contrast, the action of GLP-1(7-37) but not 10 nM glyburide was abrogated when the test solution contained no added glucose (right). Statistical significance was determined by Student's *t*-test. Probability values (\* $P \leq 0.05$ , \*\* $P \leq 0.005$ ) are expressed relative to control (10 mM glucose and no GLP-1). **c**, The number of cells tested was 50 for GLP-1(7-37) and 10 mM glucose, 25 for GLP-1(8-37) and 10 mM glucose, 20 for GLP-1(7-37) and 0 mM glucose, and 20 for glyburide and 0 mM glucose. The  $EC_{50}$  value for GLP-1(7-37) was calculated as 1 nM by establishing a cumulative dose-response relationship in which the action of the peptide was assessed over a concentration range of 0.01–100 nM. The  $EC_{50}$  was defined as the concentration of GLP-1(7-37) that depolarized 50% of the cells by  $\geq 20$  mV when the cells were simultaneously challenged with 10 mM glucose.

**METHODS.** Islets were isolated from pancreata of male rats (200–250 g) by collagenase digestion, suspended in culture medium (RPMI 1640 containing 11.1 mM glucose, 10% fetal bovine serum, 100  $\mu$ g ml $^{-1}$  streptomycin, 100 U ml $^{-1}$  penicillin G), and maintained in culture 1–4 days. Single-cell suspensions were prepared by trypsin-EDTA digestion and mechanical dispersion of cultured islets, and cells were plated on Falcon tissue culture dishes coated with concanavalin-A for short-term ( $\leq 16$  h) culture. Under these conditions,  $\geq 90\%$  of the 10–15  $\mu$ m diameter cells secrete insulin,



as determined by the reverse haemolytic plaque assay<sup>16</sup>, and are therefore considered to be  $\beta$ -cells. The fact that  $\sim 80\%$  of our cells responded to glyburide indicates that the primary rat cell cultures are indeed enriched with  $\beta$ -cells. For perforated-patch recordings the pipette solution contained 240  $\mu$ g ml $^{-1}$  nystatin and (in mM): 10 KCl, 10 NaCl, 70 K<sub>2</sub>SO<sub>4</sub>, 2 MgCl<sub>2</sub>, 10 HEPES (pH 7.35, 295 mOsm). In Figs 1–3 the bath solution contained 138 NaCl, 5.6 KCl, 1.2 MgCl<sub>2</sub>, 2.6 CaCl<sub>2</sub>, 10 HEPES (pH 7.35, 300 mOsm), and was maintained at 32 °C by a peltier device mounted on the stage of an inverted microscope. All recordings were obtained from solitary cells not organized in clusters, and recordings were initiated within 30 min after transfer of the cells to the glucose-free solution. Pipettes were prepared from borosilicate glass capillary tubes and fire polished to tip resistances of 2–8 M $\Omega$ . Measurements were made using an EPC-9 amplifier (bandwidth 10 kHz) interfaced with the Instrutech Data Acquisition and Analysis system (Instrutech Corp., Mineola, NY). The signal was stored on videotape, low-pass filtered (0.5–3.0 kHz, 4-pole Bessel filter, -3 dB attenuation), digitized (100 Hz for recordings of the membrane potential, 5–10 kHz for unitary current recordings), and selected recordings were sampled. Following seal formation, perforation was achieved within 5–10 min, at which time values for the series resistance ( $R_s$ ), slow capacitance compensation ( $C_s$ ), and the resting membrane potential were 10–20 M $\Omega$ , 6–8 pF and  $-61 \pm 5$  mV ( $n=50$ ), respectively. No correction was made for liquid junction potentials. Test substances were applied by pressure ejection from 'puffer' pipettes. The kinetics of this delivery system were established by measuring the rates of onset (time constant,  $\tau$ , 1.5 s) and offset ( $\tau$ , 10 s) of 60 mM KCl-induced depolarizations. The change in membrane potential in response to glucose or GLP-1 was measured as the difference between the resting potential and the plateau potential. GLP-1 was obtained from Scios Nova.

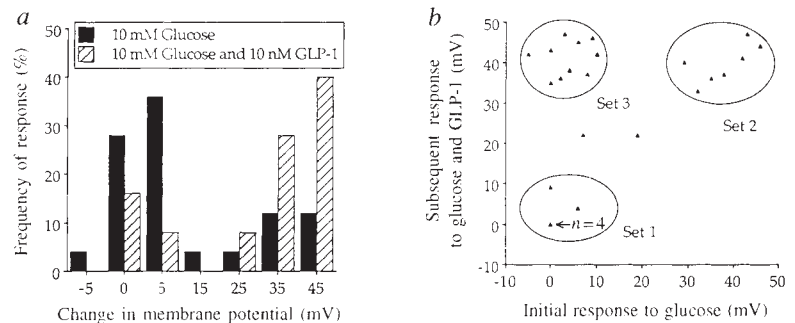
that cAMP-mediated signalling systems are necessary for the induction of glucose competence by GLP-1. Previous studies have implicated cAMP-mediated signalling systems in the regulation of the  $\beta$ -cell membrane potential<sup>25,26</sup>, possibly by controlling the activity of ATP-sensitive potassium channels ( $I_{KATP}$ ) and/or voltage-sensitive calcium channels<sup>14,27–29</sup>.

To begin to define the biophysical basis for induction of glucose competence by GLP-1, attention was focused on  $\beta$ -cells that exhibited a  $\leq 10$  mV depolarizing response when initially challenged with glucose. Voltage clamp analysis was done in the perforated patch configuration and the effects of glucose and GLP-1 on the resting membrane conductance were monitored under conditions in which the bath solution contained no glucose. Brief application of 10 mM glucose or 10 nM GLP-1 was without significant effect on membrane conductance, whereas the conductance was reversibly inhibited by combined application of both test substances (Fig. 3a). A very similar inhibitory action was also recorded during application of 10 nM glyburide (a sulphonylurea that blocks  $I_{KATP}$  with high specificity<sup>6,20</sup>) to these same cells (data not shown). These findings suggest that glucose and GLP-1 synergize to depolarize  $\beta$ -cells by closing sulphonylurea-sensitive potassium channels

( $I_{KATP}$  channels) that are open at or near the resting potential.

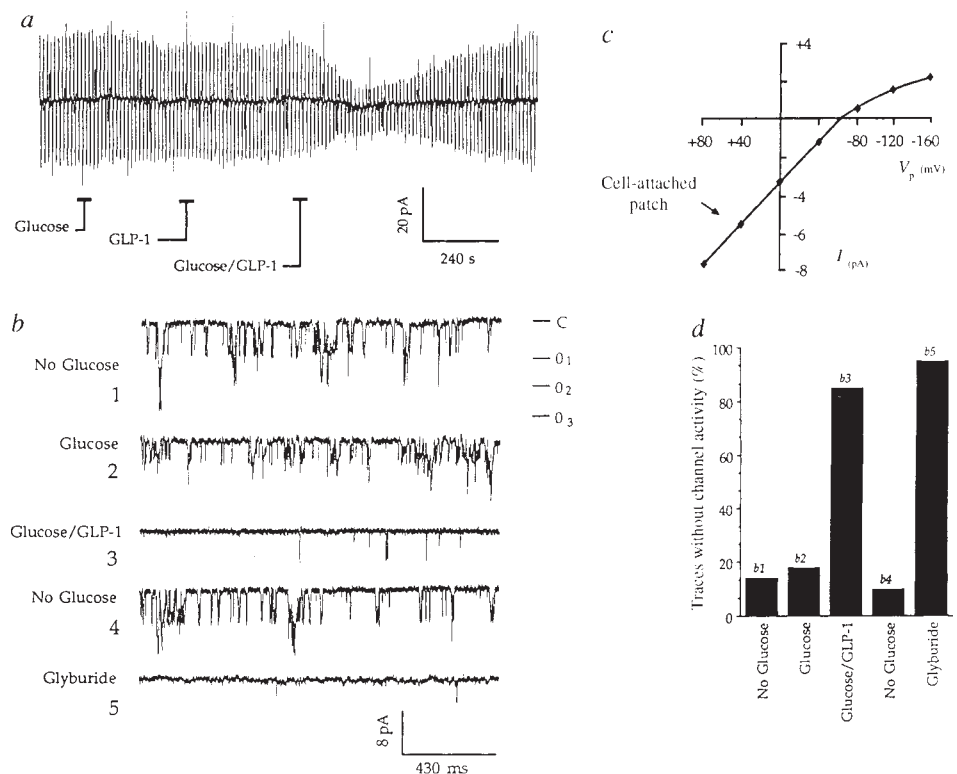
To characterize the channels inhibited by glucose and GLP-1, cell-attached patch recordings were obtained under conditions that allow analysis of inwardly-directed  $I_{KATP}$  currents. Baseline activity of single  $I_{KATP}$  channels exhibited a mean unitary current amplitude of 6.0 pA (pipette potential +50 mV) when the bath solution contained no added glucose (Fig. 3b trace 1), and the single channel conductance inferred from the unitary current amplitude as a function of voltage ( $I$ - $V$ ) relationship was 60 pS (Fig. 3c), as expected for  $I_{KATP}$ <sup>6,7</sup>. In 6 of 10 cells tested, application of 10–20 mM glucose had very little effect on channel activity (Fig. 3b trace 2), whereas a decrease in channel activity similar to that previously reported<sup>6,30</sup> was recorded from 4 cells (data not shown). When the initially glucose-insensitive cells were challenged with a combined application of 20 mM glucose and 10 nM GLP-1, the channel activity was decreased in two cells and was nearly eliminated in four cells (Fig. 3b trace 3). In addition, the unitary current amplitude decreased to 4.2 pA, as expected if glucose/GLP-1-induced whole-cell depolarization resulted in a decreased driving force for K<sup>+</sup>. Channel activity recovered (Fig. 3b trace 4) and was then blocked by glyburide (Fig. 3b trace 5), indicating that these

FIG. 2 A frequency-of-response histogram (a) and scatter plot (b) analysis summarizing the interaction of glucose and GLP-1 to depolarize  $\beta$ -cells. When initially challenged with 10 mM glucose the majority of cells exhibited a  $\leq 15$  mV depolarizing response, as indicated by either the fully shaded histogram bars in a, or the position of the triangles relative to the x-axis in b (where one triangle equals one cell except for the  $n=4$  cells triangle). When subsequently challenged with a combined application of 10 mM glucose and 10 nM GLP-1, the distribution of the histogram plot was shifted to the right (a, cross-hatched bars) and a subpopulation of  $\beta$ -cells labelled set 3 was rendered glucose competent (b, as indicated by the position of the triangles relative to the y-axis). In contrast, cells that comprised set 2 exhibited a dose-dependent depolarizing response to glucose over a concentration range of  $\sim 7$ –20 mM glucose and were constitutively glucose competent. Each illustration depicts results obtained from the same 25 cells. Test substances were applied for 30 s at 10-min intervals, and only cells exhibiting a resting membrane potential of at least  $-55$  mV were included in the data analysis. More prolonged (3–5 min) application of 10 or 20 mM glucose (without GLP-1)



to glucose-insensitive  $\beta$ -cells that comprised sets 1 and 3 did not significantly increase the magnitude of their depolarizing response. Such glucose resistance may reflect metabolic dysfunctions resulting from disaggregation of the islets and the loss of cell-to-cell contacts, or alternatively the loss of paracrine/endocrine influences that regulate glucose responsiveness.

FIG. 3 Glucose and GLP-1 synergize to decrease the resting membrane conductance and inhibit the activity of single  $I_{KATP}$  channels. a, The membrane conductance was monitored by perforated-patch recording in the voltage clamp mode under conditions in which the bath solution contained no added glucose. Application for 30 s of either 10 mM glucose or 10 nM GLP-1 was without significant effect on the magnitude of evoked current responses to  $\pm 10$  mV voltage steps from a holding potential of  $-80$  mV, whereas the evoked currents were reversibly inhibited by simultaneous application of 10 mM glucose and 10 nM GLP-1. The conductance decreased 80% from 2.0 nS to 0.4 nS and no shift in the holding current was observed. Outward currents are indicated by upward deflections. b, Cell-attached patch recordings of unitary inward currents measured when the bath solution contained no added glucose (traces 1 and 4), 20 mM glucose (trace 2), 20 mM glucose and 10 nM GLP-1 (trace 3), or 10 nM glyburide (trace 5), each applied for 30 s. Inward currents are indicated by downward deflections from a closed level (C) to three superimposed levels of openings ( $O_{1-3}$ ). Filter 1 kHz, sample rate 5 kHz. c, The  $I$ - $V$  relationship for unitary currents recorded in the cell-attached patch configuration. The slope of the  $I$ - $V$  relationship decreased at very negative pipette potentials (inward rectification), reversed direction when the pipette potential ( $V_p$ ) was more negative than  $-70$  mV, and the single channel conductance inferred from the slope of the linear portion of the  $I$ - $V$  relationship indicated a value of 60 pS, as expected for  $I_{KATP}^{6,20}$ . d, Histogram analysis summarizing the actions of glucose, GLP-1, and glyburide, as illustrated in b, traces 1–5, to inhibit  $I_{KATP}$ . The effects of these test substances were assessed by determining the frequency of occurrence of 500-ms oscilloscope traces that exhibited no channel activity (blanks). Fifty traces were recorded before, during the peak effect, and after recovery from each test substance.



for the series resistance by 80%. Glucose and GLP-1 were applied at 4-min intervals to avoid priming effects that are observed at shorter intervals. b, For cell-attached-patch recordings the pipette solution contained (in mM): 140 KCl, 5 CaCl<sub>2</sub>, 5 MgCl<sub>2</sub>, 10 HEPES (pH adjusted to 7.35 with KOH, 305 mOsm) and  $V_p$  was  $+50$  mV. Test substances were applied at 10-min intervals, and the current traces illustrated are representative of the peak effects observed. In b, trace 2, the relatively small effect of 20 mM glucose on channel activity was accompanied by a decreased unitary current amplitude, suggestive of a decreased driving force for  $K^+$ , possibly due to a depolarizing action of glucose at the whole-cell level. The decreased unitary current amplitude observed in trace 2 may therefore suggest an action of glucose to depolarize  $\beta$ -cells, not simply by inhibiting  $I_{KATP}$  channels, but also by inducing an uncharacterized conductance change occurring in the membrane outside the patch.



TABLE 1 Effects of membrane-permeant analogues of cAMP on the interaction of glucose and GLP-1(7-37) to depolarize rat pancreatic  $\beta$ -cells

| Treatment                                                 | Number of cells tested | mV Change in membrane potential ( $\pm$ s.e.m.) |
|-----------------------------------------------------------|------------------------|-------------------------------------------------|
| 10 mM glucose                                             | 10                     | 12 $\pm$ 5                                      |
| 10 mM glucose<br>10 nM GLP-1(7-37)                        | 5                      | 35 $\pm$ 10*                                    |
| 10 mM glucose<br>10 $\mu$ M Rp-cAMPS                      | 5                      | 8 $\pm$ 6                                       |
| 10 mM glucose<br>10 nM GLP-1(7-37)<br>10 $\mu$ M Rp-cAMPS | 5                      | 10 $\pm$ 7                                      |
| 10 mM glucose<br>10 $\mu$ M Sp-cAMPS                      | 5                      | 40 $\pm$ 12*                                    |

Sp- and Rp-cAMPS were obtained from BioLog Life Science Institute, La Jolla, CA and prepared as 1 mM stock solutions in distilled water. The analogues were then diluted to a final concentration of 10  $\mu$ M in the standard extracellular recording solution described in Fig. 1. Relatively glucose-insensitive  $\beta$ -cells were initially identified by testing for their inability to generate a substantial depolarizing response to 10 mM glucose applied for 30 s. After allowing 5 min recovery, such cells were subsequently challenged with a 30 s application of test solutions containing the indicated concentrations of glucose, GLP-1(7-37), and Sp-cAMPS. For those experiments examining the action of Rp-cAMPS the cells were pretreated in 10  $\mu$ M Rp-cAMPS for 30 min at 37 °C. The cells were then challenged with the indicated test solutions containing glucose, GLP-1(7-37), and Rp-cAMPS. Statistical significance was evaluated by Student's *t*-test.

\* A value that is significantly different ( $P \leq 0.05$ ) from control (10 mM glucose alone).

currents are in fact attributable to  $I_{KATP}$ . This experiment is summarized in Fig. 3d which illustrates the maximal inhibitory effects of glucose, GLP-1, and glyburide on  $I_{KATP}$  activity, expressed as the percentage of 500 ms oscilloscope traces ( $n = 50$  traces per treatment) that exhibited no channel activity (blanks). Because  $I_{KATP}$  channels within the patch were inhibited by application of glucose and GLP-1 to the membrane outside the patch, a cytosolic signalling mechanism is implicated. Whether cAMP-dependent protein phosphorylation is responsible for the inhibition of  $I_{KATP}$  will require further analysis given that conflicting reports exist concerning how phosphorylation influences the activity of these channels<sup>30-32</sup>.

The inhibitory action of glucose on  $I_{KATP}$ , as observed in the intact islet, is proposed to result from a glucose signalling system whereby uptake of glucose is mediated by a facilitative glucose transporter, intracellular glucose is converted to glucose-6-phosphate by glucokinase, and aerobic glycolysis generates ATP that is required to inhibit channel function<sup>6,7</sup>. In contrast, the best evidence available to date indicates that GLP-1 exerts its effects on  $\beta$ -cells by stimulating the production of cAMP<sup>8,21,24</sup>. The action of GLP-1 to induce glucose competence is therefore suggestive of crosstalk between the cAMP and glucose signalling systems. The consequences of crosstalk are likely to be manifest not only at the level of  $I_{KATP}$  modulation, but possibly also at more proximal components (glucose transporter, glucokinase) or distal components (voltage-sensitive calcium channels, vesicular insulin secretion) in the glucose signalling system.

These findings indicate that GLP-1, a circulating hormone, synergizes with glucose to inhibit  $I_{KATP}$ , thereby allowing  $\beta$ -cells to depolarize and generate action potentials, events that are necessary prerequisites to glucose-stimulated insulin secretion. Induction of glucose competence by GLP-1 is a receptor-mediated process that is distinct from the more direct inhibitory action of sulphonylureas on  $I_{KATP}$ , and provides a straightforward explanation for the ability of GLP-1 to stimulate insulin secretion from the pancreas. Furthermore, as is the case for its

insulinotropic action *in vivo*, the inhibition of  $I_{KATP}$  by GLP-1 exhibits an absolute requirement for glucose. It is for these reasons that GLP-1 may offer distinct therapeutic advantages over sulphonylureas when it is used for treatment of NIDDM. Administration of GLP-1 by intravenous infusion produces a rise in circulating insulin accompanied by a lowering of blood glucose, a process that is self-terminating<sup>3</sup>, as expected because the insulinotropic actions of GLP-1 are glucose-dependent. The likelihood for development of hypoglycaemia during treatment with GLP-1 is therefore reduced, and an extra margin of safety is provided that sulphonylureas or insulin do not offer. □

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- Unger R. H. & Foster, D. W. in *Williams Textbook of Endocrinology* (eds Wilson, J. D. & Foster, D. W.) 1255-1333 (Saunders, Philadelphia, 1992).
- Mojsov, S. *et al.* *J. Biol. Chem.* **261**, 11880-11889 (1986).
- Nathan, D. M., Schreiber, E., Fogel, H., Mojsov, S. & Habener, J. F. *Diabetes Care* **15**, 270-276 (1992).
- Gutniak, M., Orskov, C., Holst, J. J., Ahren, B. & Efendic, S. *New Eng. J. Med.* **326**, 1316-1322 (1992).
- Gerich, J. E. *New Eng. J. Med.* **321**, 1231-1245 (1989).
- Ashcroft, F. M. & Rorsman, P. *Prog. Biophys. molec. Biol.* **54**, 87-143 (1991).
- Rajan, A. S. *et al.* *Diabetes Care* **13**, 340-363 (1990).
- Drucker, D. J., Philippe, J., Mojsov, S., Chick, W. L. & Habener, J. F. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3434-3438 (1987).
- Mojsov, S., Weir, G. C. & Habener, J. F. *J. clin. Invest.* **79**, 616-619 (1987).
- Fehmann, H. C. & Habener, J. F. *Endocrinology* **130**, 159-166 (1992).
- Weir, G. C., Mojsov, S., Hendrick, G. K. & Habener, J. F. *Diabetes* **38**, 338-342 (1989).
- Horn, R. & Marty, A. *J. gen. Physiol.* **92**, 145-159 (1988).
- Falke, L. C., Gillis, K. D., Pressel, D. M. & Misler, S. *FEBS Lett.* **251**, 167-172 (1989).
- Smith, P. A., Ashcroft, F. M. & Rorsman, P. *FEBS Lett.* **261**, 187-190 (1990).
- Hamill, O. P., Marty, A., Neher, E., Sakman, B. & Sigworth, F. *Pflügers Arch.* **391**, 85-100 (1981).
- Hirrlinger, M. & Matteson, D. R. *J. gen. Physiol.* **91**, 617-639 (1988).
- Pipeleers, D., In't Veld, P., Maes, E. & Van De Winkel, M. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7322-7325 (1982).
- Sherman, A., Rinzel, J. & Keizer, J. *Biophys. J.* **54**, 411-425 (1988).
- Wang, J., Baimbridge, K. G. & Brown, J. C. *Endocrinology* **131**, 146-152 (1992).
- Ashcroft, F. M. *A. Rev. Neurosci.* **11**, 97-118 (1988).
- Blackmore, P. F., Mojsov, S., Exton, J. H. & Habener, J. F. *FEBS Lett.* **283**, 7-10 (1991).
- Goke, R. & Conlon, J. M. *J. Endocrinol.* **116**, 357-362 (1988).
- Fehmann, H. C. & Habener, J. F. *Endocrinology* **128**, 2880-2888 (1991).
- Thorens, B. *Proc. natn. Acad. Sci. U.S.A.* **89**, 8641-8645 (1992).
- Henquin, J. C. & Meissner, H. P. *Endocrinology* **115**, 1125-1134 (1984).
- Eddelstone, G. T., Oldham, S. B., Lipson, L. G., Premdas, F. H. & Beigelman, P. M. *Am. J. Physiol.* **248**, C145-C153 (1985).
- Smith, P. A., Rorsman, P. & Ashcroft, F. M. *Nature* **342**, 550-553 (1989).
- Prektil, M., Glennon, M. C., Geschwind, J. F., Matschinsky, F. M. & Corkey, B. E. *FEBS Lett.* **220**, 103-107 (1987).
- Rajan, A. S., Hill, R. S. & Boyd, A. E. *Diabetes* **38**, 874-880 (1989).
- Ribalet, B., Ciani, S. & Edelstone, G. T. *J. gen. Physiol.* **94**, 693-717 (1989).
- Nichols, C. G. & Lederer, W. J. *J. gen. Physiol.* **97**, 1095-1098 (1991).
- Wang, W. & Giebisch, G. *Proc. natn. Acad. Sci. U.S.A.* **88**, 9722-9725 (1991).

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## Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA

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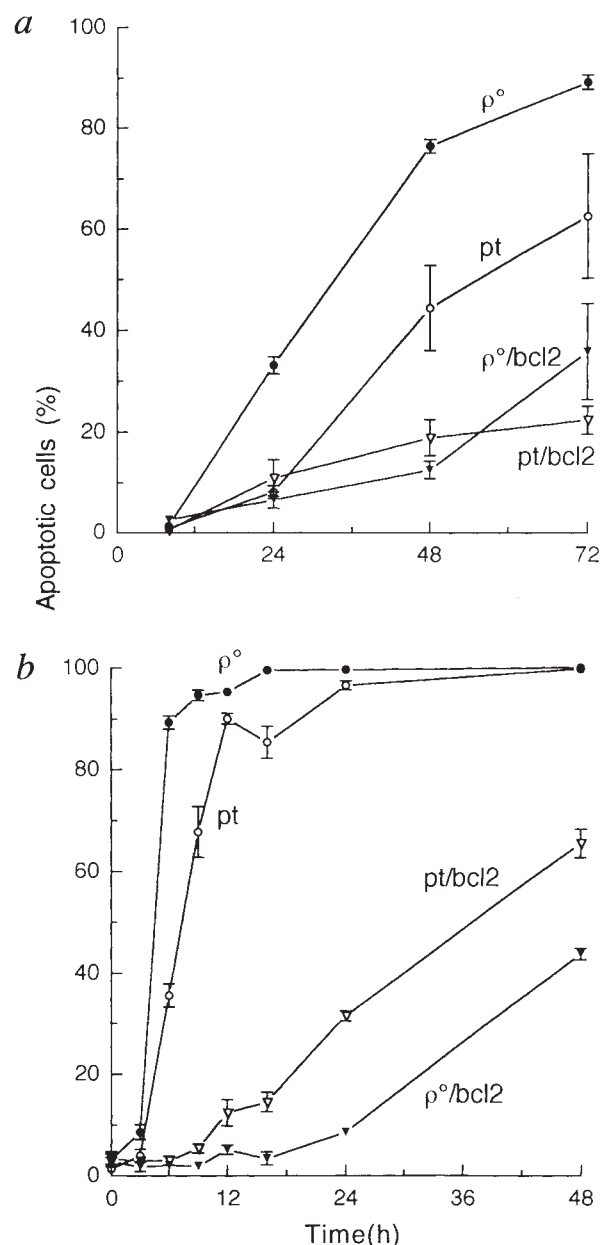
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WHEN the mammalian proto-oncogene *bcl-2* is overexpressed it can protect various types of cells both from normal and from experimentally induced apoptosis<sup>1-6</sup>, but the molecular mechanisms involved are unknown. Although the Bcl-2 protein is membrane-associated<sup>7-10</sup>, its subcellular location is controversial: two studies have suggested that it is mainly associated with the nuclear envelope and endoplasmic reticulum<sup>8,10</sup>, whereas another study has suggested that it is mainly located in the inner mitochondrial membrane<sup>9</sup>. The latter study has suggested that Bcl-2 might protect cells from apoptosis by altering mitochondrial function and that

FIG. 1 Apoptotic cell death of parental (pt) and mutant ( $\rho^0$ ) cells, and of *bcl-2*-overexpressing parental (pt/*bcl-2*) and mutant ( $\rho^0$ /*bcl-2*) cells after withdrawal of FCS (a) or treatment with 1  $\mu$ M staurosporine (b).

METHODS. The  $\rho^0$ 701.2a cell line<sup>18,24</sup> ( $\rho^0$  cells) was derived from a simian virus 40 T-antigen-gene-transformed derivative of the human fibroblast line GM701 (ref. 25) (pt cells) by long-term exposure to low concentrations of ethidium bromide (M.P.K. and G. Attardi, unpublished data). These cells completely lack mitochondrial DNA, are unable to carry out cellular respiration, require pyruvate (110  $\mu$ g ml<sup>-1</sup>) and uridine (50  $\mu$ g ml<sup>-1</sup>) to grow<sup>13</sup>, and their mitochondria appear abnormal in electron micrographs (our unpublished observations). *Bcl-2*-overexpressing cell lines were isolated by selecting G418-resistant clones of pt and  $\rho^0$  cells that were infected with Zip-*bcl-2* (ref. 26) (T.M. and J.C.R., manuscript in preparation). Three independent transfected  $\rho^0$  cell lines were isolated and studied. The results with one of these *Bcl-2*-expressing subclones are shown; in similar independent experiments, the behaviour of the other subclones was indistinguishable. To assay for apoptosis, cells were removed from culture dishes with trypsin, EDTA, washed once in DMEM, 10%FCS, resuspended in DMEM, and then plated on poly-D-lysine-coated 13 mm glass coverslips in 24-well Falcon plates at a density of 10,000 cells per well. a, The cells were plated in serum-free DMEM (time = 0). b, The cells were cultured in DMEM, 10%FCS and staurosporine was added at various times. After the treatment period (time after plating for serum withdrawal (a) or time after staurosporine addition (b)), the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PB) for 10 min at room temperature, followed by acid-alcohol for 10 min at -20 °C, and then stained with propidium iodide (0.05  $\mu$ g ml<sup>-1</sup> in phosphate-buffered saline) containing 100  $\mu$ g ml<sup>-1</sup> DNase-free RNase A for 30 min at 37 °C to visualize the nuclei<sup>27</sup>. The cells were mounted in Citifluor (City University, London) and examined with a Zeiss Universal fluorescence microscope and photographed with Tri-X film rated 400 ASA. Apoptotic nuclei were easily distinguished from normal nuclei; they were condensed, brightly fluorescent and often fragmented (see Fig. 2b, c). The results shown are means  $\pm$  s.e.m. of quadruplicate measurements in one experiment, but similar results were obtained in at least three independent experiments. For all cell types, the proportion of apoptotic cells after 48 and 72 h was less than 4% when cultured in DMEM, 10%FCS.



mitochondria may be involved in apoptosis<sup>9,11</sup>. Here we report that human mutant cell lines that lack mitochondrial DNA (mtDNA), and therefore do not have a functional respiratory chain, can still be induced to die by apoptosis, and that they can be protected from apoptosis by the overexpression of *bcl-2*, suggesting that neither apoptosis nor the protective effect of *bcl-2* depends on mitochondrial respiration. We also show that the *Bcl-2* protein in overexpressing cells is associated with the nuclear envelope and endoplasmic reticulum, as well as with mitochondria.

We studied a human fibroblast cell line that was entirely depleted of mtDNA by long-term exposure to low concentrations of ethidium bromide as previously described<sup>12,13</sup>. Although nearly all of the proteins located in the mitochondria are encoded in the nuclear genome and imported from the cytosol, 13 polypeptide components of the multisubunit enzyme complexes of the electron-transport chain present in the inner mitochondrial membrane are encoded by the mitochondrial genome<sup>14</sup>. Therefore, in cells that have no mtDNA (termed  $\rho^0$  cells), the electron-transport chain cannot function and the cells cannot carry out oxidative phosphorylation. Consequently, these mutant cells rely exclusively on glycolysis for their energy requirements and require exogenous pyruvate and pyrimidines for growth<sup>12,13</sup>.

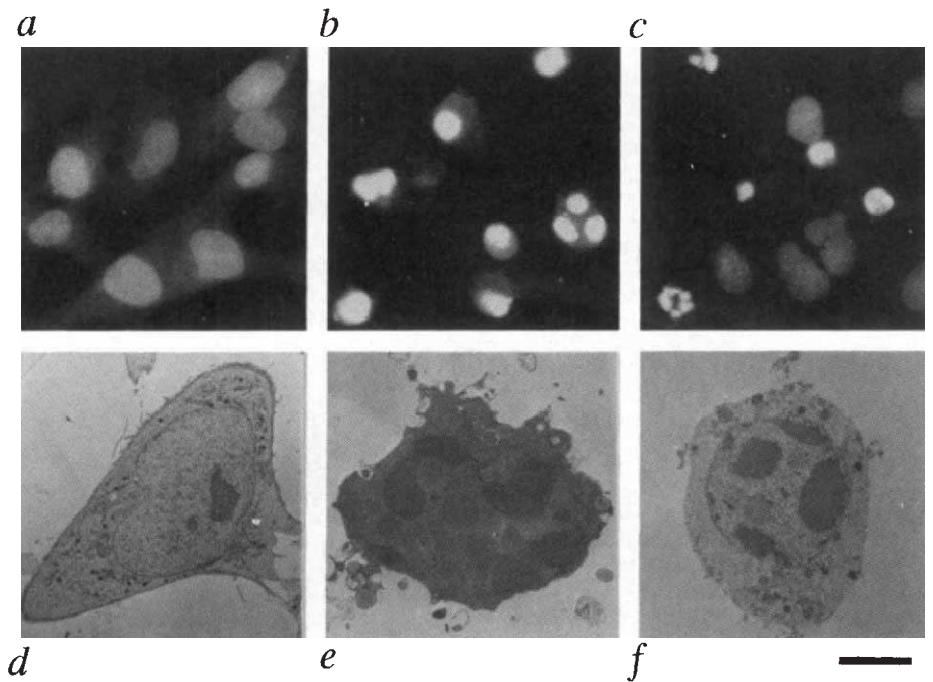
Accordingly, the  $\rho^0$  cells and, for convenience, the parental line from which the mutant line was derived, were maintained in DMEM supplemented with 10% fetal calf serum (FCS), pyruvate and uridine<sup>13</sup>.

Many cell types undergo apoptosis when they are deprived of serum or specific growth factors (reviewed in ref. 15), and this was the case both for the parental and for the  $\rho^0$  cells (Fig. 1a). When grown in the absence of FCS, many of the cells died asynchronously over several days, with morphological features that are characteristic of apoptosis<sup>16</sup>: when viewed by time-lapse video recording, the cells showed active surface blebbing and then shrank and often fragmented (not shown), and when stained with propidium iodide or viewed in an electron microscope, the nuclei of the dead cells were condensed and often fragmented (Fig. 2c, f). A similar proportion of parental and  $\rho^0$  cells died under these conditions (Fig. 1a); in both cases, the proportions of apoptotic cells were greater than those shown in the figure, as many of the dead cells detached from the substratum and therefore were not counted.

High concentrations of the protein kinase inhibitor staurosporine will induce many cell types in culture to undergo apoptosis, perhaps because it blocks the intracellular signalling



FIG. 2 Propidium iodide fluorescence (*a-c*) and electron microscopy (*d-f*) of  $\rho^0$  cells cultured in 10% FCS for 48 h (*a, d*), in 10% FCS and 1  $\mu$ M staurosporine for 18–20 h (*b, e*), or in the absence of FCS for 48 h (*c, f*). The dead cells in *b, c* and *e, f* show characteristic features of apoptosis. Note that the apoptotic cells in *e, f* are about three times smaller in diameter than the normal cell in *d*. Scale bars, 25  $\mu$ m in *a-c*, 10  $\mu$ m in *d* and 3–4  $\mu$ m in *e, f*. METHODS. Cells were fixed, stained with propidium iodide and photographed as described in Fig. 1. For electron microscopy, the cells were fixed in 2% glutaraldehyde in PB for 45 min at 37 °C, post-fixed in 1% osmium tetroxide for 30 min at 4 °C, stained in uranyl acetate, dehydrated through graded ethanol and embedded in Epon 812 resin. Thin sections were cut parallel to the substratum, stained with uranyl acetate and lead citrate, and examined with a Jeol 100 CXII electron microscope at 80 kV.



pathways activated by the extracellular survival factors that many cells require to live<sup>15</sup>. As shown in Figs 1*b* and 2*b* and *e*, more than 95% of both the  $\rho^0$  and the parental cells underwent apoptosis within 24 h when treated with 1  $\mu$ M staurosporine in the presence of 10% FCS. Thus cells without mtDNA can undergo apoptosis when either deprived of survival factors or exposed to high concentrations of staurosporine.

To determine whether the overexpression of *bcl-2* could protect  $\rho^0$  cells from apoptosis induced by FCS withdrawal or staurosporine treatment, we introduced a human *bcl-2* cDNA into  $\rho^0$  and parental cells using a retroviral vector. The vector also contained the selectable marker *neo<sup>r</sup>*, which enabled us to select for G418-resistant, *bcl-2*-expressing subclones. Each of three independent, stably transfected  $\rho^0/bcl-2$  cell lines was found to express high levels of Bcl-2 protein, as determined by staining with a monoclonal anti-Bcl-2 antibody<sup>17</sup> (see below), and to be relatively resistant to the lethal effects of serum deprivation (Fig. 1*a*) or staurosporine treatment (Fig. 1*b*). We also tested an unrelated human osteosarcoma-derived  $\rho^0$  cell line ( $\rho^0$ 13.1; M.P.K. and G. Attardi, unpublished observations, and ref. 18), and it behaved in the same way: the cells could be induced to undergo apoptosis by either serum deprivation or staurosporine treatment, and they were protected by transfection with *bcl-2* (not shown). When  $\rho^0$  cells were acutely infected with the *bcl-2*-expressing retrovirus, only about 5% of the cells seemed to be successfully transfected, as judged by staining with the anti-Bcl-2 antibody; after treatment with staurosporine, the small number of cells that survived were all intensely stained by the antibody (not shown). Thus *bcl-2* can protect cells from undergoing apoptosis even if the cells lack a functional respiratory chain.

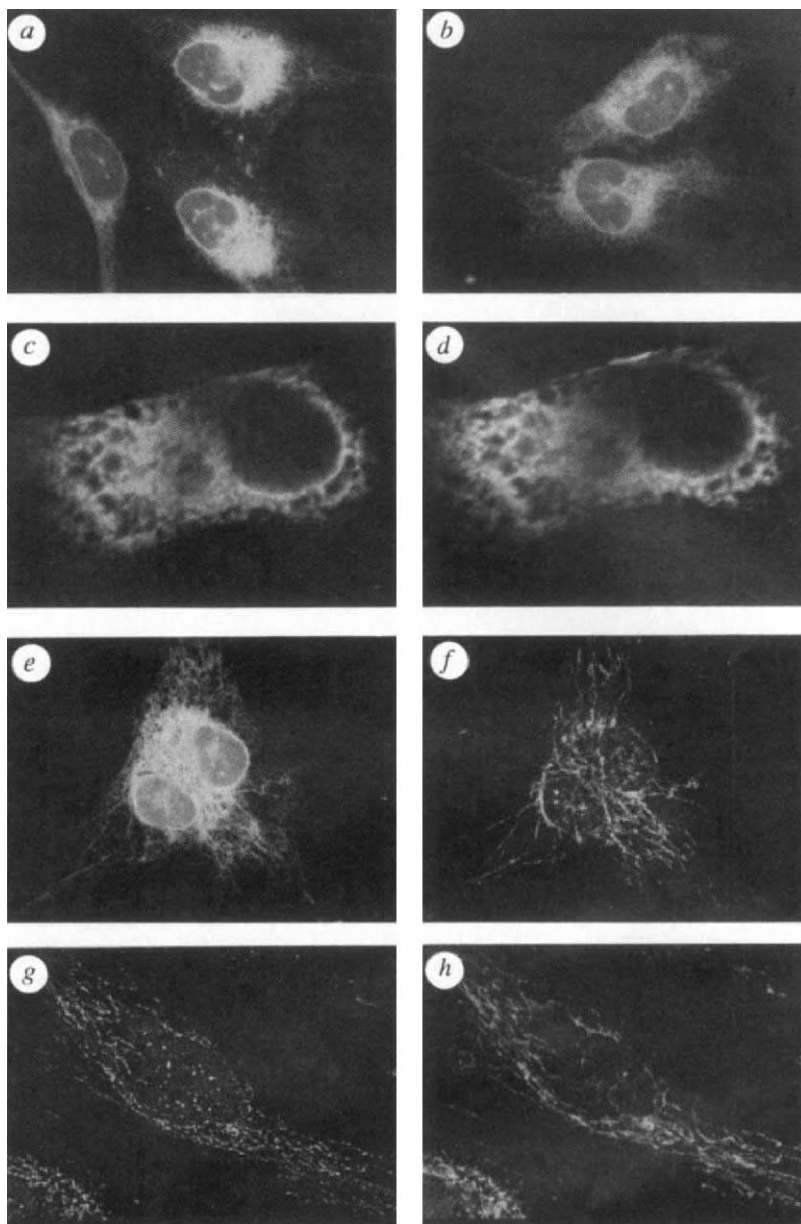
To be certain that the  $\rho^0$  and  $\rho^0/bcl-2$  cells that we studied lacked a functional respiratory chain, we tested their respiratory function in two ways. First, we showed that, unlike the parental cells, the  $\rho^0$  cells were unable to proliferate in medium lacking pyrimidines: when  $10^7$   $\rho^0$  cells were plated in 20 culture dishes, no colonies were detected after 4 weeks (data not shown). Using identical conditions, it has been shown that injection of a single mitochondrion (containing ~10 mtDNA molecules) into  $\rho^0$  cells results in the complete repopulation of the recipient  $\rho^0$  cells with mtDNA and permits their subsequent proliferation in the absence of pyrimidines<sup>13</sup>. Second, we examined *bcl-2*-transfected and untransfected parental (pt) and  $\rho^0$  cells for their rates

of oxygen consumption (as previously described<sup>19</sup>) and found that the  $\rho^0$  and  $\rho^0/bcl-2$  cells respired at a rate less than 3% of that of the pt cells. The values (fmol O<sub>2</sub> per cell per min, means  $\pm$  s.e.m.,  $n = 8$ ) were: pt =  $4.12 \pm 0.09$ , pt/*bcl-2* =  $4.51 \pm 0.23$ ,  $\rho^0$  =  $0.10 \pm 0.01$  and  $\rho^0/bcl-2$  =  $0.08 \pm 0.01$ . These results also show that the overexpression of *bcl-2* does not significantly alter the levels of mitochondrial respiratory chain activity in either the parental or the  $\rho^0$  cells.

When the subcellular distribution of the Bcl-2 protein was analysed in the  $\rho^0/bcl-2$  or pt/*bcl-2* cells lines by immunofluorescence with a mouse monoclonal anti-Bcl-2 antibody<sup>17</sup> and laser-scanning confocal microscopy, the protein seemed to be located mainly in the nuclear envelope and endoplasmic reticulum (ER) (Fig. 3*a, b*). When, however, the cells were double-labelled with the anti-Bcl-2 antibody and either human autoantibodies against a set of mitochondrial proteins<sup>20,21</sup> or a rabbit antiserum against ER proteins<sup>22</sup>, it could be seen that Bcl-2 was present in the mitochondria (Fig. 3*e, f*), as well as in the nuclear envelope and ER (Fig. 3*c, d*). Although the anti-Bcl-2 antibody did not label the plasma membrane, we cannot exclude the possibility that it labelled other organelles in addition to the nuclear envelope and ER. The pattern of labelling with the anti-Bcl-2 antibody was the same in *bcl-2*-transfected parental and  $\rho^0$  cells, whereas untransfected cells were not labelled above background (not shown). In preliminary studies, we have found that in primary rat glial and neuronal cells that were transfected with human *bcl-2*, the anti-Bcl-2 antibody also seemed to stain the nuclear envelope and ER (M.D.J. and J. Voyvodic, unpublished data). In a recent independent study, Alnemri *et al.* found Bcl-2 mainly in the nuclear envelope and ER of insect cells that had been transfected with a human *bcl-2* complementary DNA<sup>10</sup> (consistent with an earlier study by Liu *et al.*, which found endogenous Bcl-2 localized to both mitochondrial and non-mitochondrial sites of human tonsillar B cells<sup>23</sup>). Thus, at least in cells that overexpress *bcl-2*, the Bcl-2 protein is not confined to mitochondria.

We have shown the following: (1) cells without mtDNA, and therefore lacking respiratory chain activity, are still able to undergo apoptosis when either deprived of survival factors or exposed to high concentrations of staurosporine; (2) such cells can be protected from apoptosis by the overexpression of *bcl-2*; (3) the overexpression of *bcl-2* in cells (with or without mtDNA) does not significantly alter respiratory chain activity; (4) the

FIG. 3 Laser-scanning confocal fluorescence micrographs of *bcl-2*-transfected parental (a, c–h) and *p<sup>0</sup>* (b) cells, showing the distribution of the Bcl-2 protein (a, b, c, e), ER proteins (d) and mitochondrial proteins (f–h). c, d, Paired images of Bcl-2 and ER proteins in the same field, whereas e, f are paired images of Bcl-2 and the mitochondrial proteins in the same field. g, h, Paired images of mitochondrial proteins in the same cell labelled with two different antimitochondrial antibodies to verify the mitochondrial specificity of the antibody shown in f; the same antibodies were used in f and g. Note that the nuclear envelope, ER and mitochondria are labelled by the anti-Bcl-2 antibody. Scale bar, 20  $\mu$ m in a, b, e–h and 10  $\mu$ m in c, d.



**METHODS.** In a, b, e–h, the cells were fixed in methanol for 10 min at  $-20^{\circ}\text{C}$ . In c, d, they were fixed in 4% paraformaldehyde in PB for 10 min and then permeabilized in 0.1% Triton X-100 for 15 min, both at room temperature. After washing, the cells were incubated in 50% normal goat serum (NGS) for 30 min at room temperature. Bcl-2 was labelled with a mouse IgG1 monoclonal anti-human Bcl-2 antibody<sup>17</sup> (supernatant diluted 1:1), which was visualized in a, b and e with a fluorescein-coupled sheep anti-mouse immunoglobulin (Sh anti-Mlg-FI, Jackson Laboratories, diluted 1:100), and in c with biotin-coupled sheep anti-Mlg (Sh anti-Mlg BT) followed by fluorescein-coupled streptavidin (SA-FI) (both from Amersham, diluted 1:100). ER proteins were labelled with a rabbit polyclonal antiserum against rough ER proteins<sup>22</sup> (diluted 1:100). The antibody was visualized with Texas-red-coupled goat anti-rabbit immunoglobulin (Jackson Laboratories; diluted 1:100). Mitochondrial proteins were labelled with an autoimmune serum from a patient with primary biliary cirrhosis<sup>20,21</sup> (The Binding Site; diluted 1:200) and visualized with a sheep anti-human Ig-FI (Wellcome, diluted 1:50). The mitochondrial specificity of the human autoantibodies was verified by double-labelling pt/*bcl-2* cells with these autoantibodies (g) and a mouse monoclonal antibody against subunit IV of cytochrome c oxidase<sup>28</sup> (h, ascites fluid diluted 1:100) visualized with Texas-red-coupled goat anti-mouse immunoglobulin (Jackson Laboratories; diluted 1:100). All of the antibodies were diluted in 1% BSA, 100 mM L-lysine in Tris-buffered saline containing 0.04% sodium azide, and incubations with primary antibodies were for 2 h and with secondary antibodies and SA for 1 h, all at room temperature. In c–f, the anti-Bcl-2 antibody was used simultaneously with the anti-ER or anti-mitochondrial protein antibodies. Under these conditions there was no significant crossreaction of the conjugates to the inappropriate antibodies. Moreover, no staining was seen when the primary antibodies were omitted or when the anti-Bcl-2 antibody was replaced with an IgG1 monoclonal antibody against bromodeoxyuridine<sup>29</sup> (culture supernatant, diluted 1:1). Similar results were obtained when a rabbit anti-biphosphorin I antiserum<sup>30</sup> or immunoadsorbant-purified rat IgG2a monoclonal antibodies against the KDEL tetrapeptide<sup>31,32</sup> were used to label the ER in double-labelling experiments with anti-Bcl-2 antibody (not shown). The cells were mounted in Citifluor with added 1.5% (w/v) *N*-propylgallate to help overcome the fading of Texas

red on the confocal microscope. Cells were examined using a Biorad MRC 600 scanning confocal microscope equipped with an argon-xenon laser and printed from the computer using a Mitsubishi colour video copy processor.

Bcl-2 protein in these cells is associated with the nuclear envelope and ER, as well as with mitochondria. Taken together, these findings exclude the possibility that either apoptosis or the protective effect of *bcl-2* involves the mitochondrial respiratory chain, at least in these cells under the conditions that we have studied, and raise the possibility that the Bcl-2 protein might exert its protective effect while bound to the continuous nuclear and ER membrane. □

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- Vaux, D. L., Cory, S. & Adams, J. M. *Nature* **335**, 440–442 (1988).
- McDonnell, T. J. *et al. Cell* **57**, 79–88 (1989).
- Núñez, G. *et al. J. Immunol.* **144**, 3602–3610 (1990).
- Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O. & Korsmeyer, S. J. *Cell* **67**, 879–888 (1991).
- Strasser, A., Harris, A. W. & Cory, S. *Cell* **67**, 889–899 (1991).
- Garcia, I., Martinou, I., Tsujimoto, Y. & Martinou, J.-C. *Science* **258**, 302–304 (1992).

- Tsujimoto, Y., Ikegaki, N. & Croce, C. M. *Oncogene* **2**, 3–7 (1987).
- Chen-Levy, Z., Nouse, J. & Cleary, M. L. *Molec. cell. Biol.* **9**, 701–710 (1989).
- Hockenbery, D. M., Núñez, G., Millman, C., Schreiber, R. O. & Korsmeyer, S. J. *Nature* **348**, 334–336 (1990).
- Alnemri, E. S., Robertson, N. M., Fernandes, T. F., Croce, C. M. & Litwack, G. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7295–7299 (1992).
- Korsmeyer, S. J. *Immun. Today* **13**, 285–288 (1992).
- Desjardins, P., Frost, E. & Morais, R. *Molec. cell. Biol.* **5**, 1163–1169 (1985).
- King, M. P. & Attardi, G. *Science* **246**, 500–503 (1989).
- Attardi, G. & Schatz, G. A. *Rev. Cell. Biol.* **41**, 278–333 (1988).
- Raff, M. C. *Nature* **356**, 397–400 (1992).
- Wyllie, A. H., Kerr, J. F. R. & Currie, A. R. *Int. Rev. Cytol.* **68**, 251–305 (1980).
- Pezzella, F. *et al. Am. J. Path.* **137**, 225–232 (1990).
- Chomyn, A. *et al. Molec. cell. Biol.* **11**, 2236–2244 (1991).
- King, M. P., Koga, Y., Davidson, M. & Schon, E. A. *Molec. cell. Biol.* **12**, 480–490 (1992).
- Sudoyo, H., Marzuki, S., Trounce, I. & Byrne, E. J. *neural. Sci.* **98**, 185–193 (1990).
- Bassendine, M. F. & Yeaman, S. J. *Hepatology* **15**, 545–548 (1992).
- Louvard, D., Reggio, H. & Warren, G. J. *Cell Biol.* **92**, 92–107 (1982).
- Liu, Y. J. *et al. Eur. J. Immun.* **21**, 1905–1910 (1991).
- Attardi, G. *et al. in Progress in Neuropathology* (eds Sato, T. & DiMauro, S.) 75–92 (Raven, New York, 1991).
- Fung, C. H. & Khachadurian, A. K. *J. Biol. Chem.* **255**, 676–680 (1980).
- Cepko, C. L., Roberts, B. E. & Mulligan, R. C. *Cell* **37**, 1053–1062 (1984).



27. Barres, B. A. *et al. Cell* **70**, 31–46 (1992).  
 28. Nakagawa, M. & Miranda, A. F. *Exp Cell Res* **168**, 44–52 (1987).  
 29. Magaud, J. P., Sargent, I. & Mason, D. Y. *J. Immun. Meth.* **106**, 95–100 (1988).  
 30. Hartsch, M. & Meyer, D. I. *Eur. J. Biochem.* **150**, 559–564 (1985).  
 31. Napier, R. M. *et al. Planta* **176**, 519–526 (1988).  
 32. Napier, R. M. *et al. J. Cell Sci.* **102**, 261–271 (1992).

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## Resistance to cadmium mediated by ubiquitin-dependent proteolysis

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**CADMIUM** is a potent poison for living cells. In man, chronic exposure to low levels of cadmium results in damage to kidneys and has been linked to neoplastic disease and ageing, and acute exposure can cause damage to a variety of organs and tissues<sup>1</sup>. Cadmium reacts with thiol groups and can substitute for zinc in certain proteins<sup>2</sup>, but the reason for its toxicity *in vivo* remains uncertain. In eukaryotes, an important selective proteolysis pathway for the elimination of abnormal proteins that are generated under normal or stress conditions is ATP-dependent and mediated by the ubiquitin system<sup>3–5</sup>. Substrates of this pathway are first recognized by ubiquitin-conjugating enzymes<sup>5–7</sup> (or auxiliary factors) which covalently attach ubiquitin, a small and highly conserved protein, to specific internal lysine residues of proteolytic substrates. Ubiquitinated substrates are then degraded by the proteasome, a multisubunit protease complex<sup>8–10</sup>. Here we show that expression of this ubiquitin-dependent proteolysis pathway in yeast is activated in response to cadmium exposure and that mutants deficient in specific ubiquitin-conjugating enzymes are hypersensitive to cadmium. Moreover, mutants in the proteasome are hypersensitive to cadmium, suggesting that cadmium resistance is mediated in part by degradation of abnormal proteins. This indicates that a major reason for cadmium toxicity may be cadmium-induced formation of abnormal proteins.

Several genes encoding distinct ubiquitin-conjugating enzymes have been cloned and shown to mediate strikingly different cellular functions<sup>5,7</sup>. We have cloned the gene *UBC7* from the yeast *Saccharomyces cerevisiae* which encodes a ubiquitin-conjugating enzyme (*M*, 18.2K, 165 amino acids) with significant sequence similarity to yeast UBC1 (ref. 11) and the closely related enzymes UBC4 and UBC5 (ref. 12) (Fig. 1). *ubc7* mutants generated by gene disruption are viable and grow at wild-type rates, indicating that UBC7-mediated functions are neither required nor important under normal growth conditions. Unlike other *ubc* mutants<sup>11–13</sup>, *ubc7* mutants are not heat-shock sensitive, hypersensitive to amino-acid analogues, or vulnerable to DNA-damaging agents.

As cadmium impairs protein structure *in vitro*<sup>2</sup>, we investigated the effect of cadmium on *ubc7* mutant cells. We found that *ubc7* mutants are cadmium-hypersensitive (Fig. 2a). In the presence of 30  $\mu$ M cadmium, *ubc7* mutants failed to form

a

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-120 attacagcctctaacaagagctcgaaaagcccaagtaaaactctggcggtttagcgta
-60 cgaaggagattatcctaaaaggaacttccctagtaaatgtgtaatttgggaaggatcagc
1 ATGTGCGAAACCGCTCAGAAAGCTCTCCTCAAGGAGCTTCAACAGTTAATTAAGATTCT
MetSerLysThrAlaGlnLysArgLeuLeuLysGluLeuGlnGlnLeuLysAspSer
61 CCACCTGGTATAGTGGTGGTCCCAATCGGAGAATAACATATTTCATTGGGACTGCCTA
ProProGlyIleValAlaGlyProLysSerGluAsnAsnIlePheIleTrpAspCysLeu
121 ATTCAAGGCGCTCCAGATACGCCATACGCTGATGGTGTGTTTAAATGCTAAGCTAGAGTTT
IleGlnGlyProProAspThrProTyrAlaAspGlyValPheAsnAlaLysLeuGluPhe
181 CCTAAGACTATCCGTTATCTCCACCTAACTTACTTTCACACCCAGCATACTACATCCA
ProLysAspTyrProLeuSerProProLysLeuThrPheThrProSerIleLeuHisPro
241 AATATTATCCCAATGGGGAAGTGTGCATATCCATTCTACACTCCCTGGTGATGATCCT
AsnIleTyrProAsnGlyGluValCysIleSerIleLeuHisSerProGlyAspAspPro
301 AACATGTACGAATTAGCGGAAGAAGATGGTCGCCAGTGCAGAGTGTAGAAAAATCTTA
AsnMetTyrGluLeuAlaGluGluArgTrpSerProValGlnSerValGlnLysLeuLeu
361 TTAAGTGTATGAGCATGTTGAGTGAGCCCAATATCGAAAGTGGTCCCAACATGATGCT
LeuSerValMetSerMetLeuSerGluProAsnIleGluSerGlyAlaAsnIleAspAla
421 TGCATCTTGTGGAGAGATAATAGACCTGAATTTGAGAGACAGGTAAGTTATCCATTTTG
CysIleLeuTrpArgAspAsnArgProGluLysGluArgGlnValLysLeuSerIleLeu
481 AAATCATTAGGATTCTGAAGcaggttagcaggttaaatgatcatttggtcttctctttaac
LysSerLeuGlyPhe***
541 tgttctctatatacataaataagcatatctaccatctaaagaaagcaaatgtcacataa
601 taaatatgttataa
  
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b

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UBC7 M S K T A Q K R I L K E C Q Q L I K D S P G I V A G G K F R N T F I X Q I I I Q G P P T P Y A
UBC1 M S P A - - K R I N K E I Q A V K D G P A A H I C L E F V D F S H L S K G T F L G P P S P Y K
UBC4 M S S S - - K R I A K E I S D C I P R D P F T S C A G P V G U - D Y Y H D A S I I H I P A D S P Y A

UBC7 D G V F N A K I K P P K D Y P L S P R L T P F S I L R F N I Y - C G V C I S I L H S P G D D
UBC1 D G K P V V D I K V P K C T P F K P P K M Q F D T V Y H N I S S V T G A I C I D I L K - - - -
UBC4 D G V F P I S I N F P T D Y P P F K P K T P T K I Y H N I N A - N G K I C L I I K - - - -

UBC7 P M Y S L A E K R P V V G S V E R I C S V R K I H I R I V I I A S S Q A T C K R N R P
UBC1 - - - - - N A W S P V I F L S A I C S I P A L I Q P I S S V T G A I C I D I L K - - - -
UBC4 - - - - - D Q W S P A L T S K V L S I C I L I L T D A V P P F L Y P K I A R I Y K T U R P

UBC7 E P F I R Q V K I I C I D S L G P
UBC1 S P K X T A A I T R I Y A S E T S N G Q R N V R E S D Y G I D H D I E D F K S Q G E K D K
UBC4 K Y I A T A R P K T K E Y A V

UBC1 I V E V L K R I G V R S D P N O N T A N R I I E A L Y
  
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**FIG. 1** Primary structure of UBC7. *a*, Nucleotide sequence of the *UBC7* gene and predicted amino-acid sequence of the encoded protein. The putative active-site cysteine residue of UBC7 required for thiolester formation with ubiquitin is underlined. Nucleotide numbers starting at the first nucleotide of the coding region are given on the left. Asterisks represent the stop codon. *b*, Amino-acid sequence similarity of UBC7 to yeast UBC1 and UBC4 enzymes. Amino acids of UBC7 identical to UBC1 (28% identity) or UBC4 (35% identity) proteins are boxed. The similarity to UBC5 is not shown as UBC5 differs in only 11 amino acids from the related UBC4 enzyme<sup>12</sup>. The putative active-site cysteine residues are located at conserved positions and marked in bold. Yeast UBC7 also shares 45% identical amino acids with a wheat enzyme<sup>20</sup>, suggesting that both proteins are functionally similar. **METHODS.** *UBC7* was fortuitously cloned from a YEp24-based library in an unrelated study<sup>21</sup>. A larger clone was isolated from a  $\lambda$ EMBL3a yeast genomic DNA library (R. Young, Whitehead Institute) by hybridization with the original insert. Restriction fragments were cloned into M13 vectors for DNA sequencing.

colonies on agar plates. Growth was reduced at 20  $\mu$ M cadmium, whereas at 10  $\mu$ M cadmium *ubc7* mutants behaved like wild-type cells (results not shown). Interestingly, *ubc7* mutants are not hypersensitive to copper, zinc or lead, suggesting that the UBC7 enzyme functions primarily in cadmium-resistance pathways. We then tested whether cadmium hypersensitivity is specific for *ubc7* mutants or if cadmium hypersensitivity can also be observed with strains mutated in genes encoding other ubiquitin-conjugating enzymes. In fact, *ubc1* and *ubc4* mutants showed cadmium sensitivities similar to *ubc7* mutants (data not shown). Previously, *ubc1* and *ubc4* mutants (*ubc5* mutants have no mutant phenotype as long *UBC4* is present) were shown to be hypersensitive to amino-acid analogues and defective in major protein degradation pathways<sup>11,12</sup>, suggesting that the observed cadmium sensitivities of *ubc* mutants are due to defects in the degradation of abnormal proteins.

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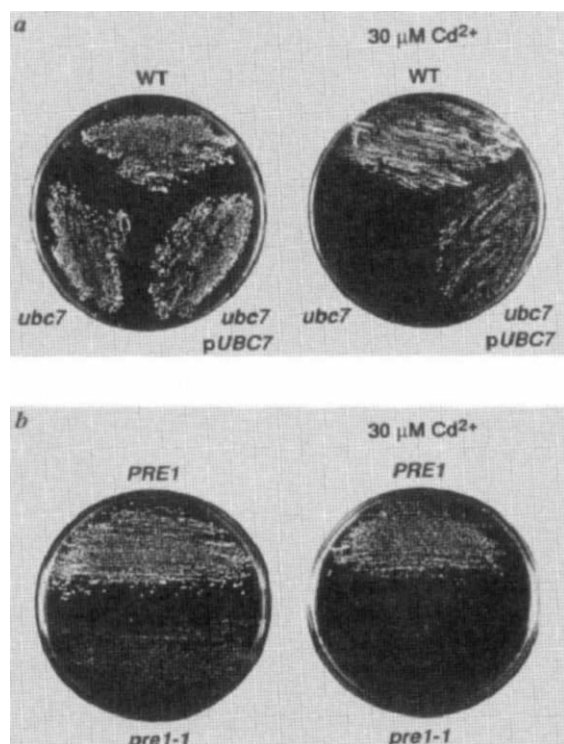


FIG. 2 Cadmium hypersensitivities of mutants of the ubiquitin-dependent proteolysis system. *a*, *ubc7* mutants are cadmium-hypersensitive. Wild-type (WT), *ubc7* mutants (*ubc7*) and *ubc7* mutant cells transformed with a 2- $\mu$ -based plasmid bearing the wild-type *UBC7* gene (1.1 kb *UBC7* fragment; *ubc7/pUBC7*) were streaked out on selection plates with or without 30  $\mu$ M cadmium (as chloride) and incubated for 5 days at 30  $^{\circ}$ C to determine colony formation. *b*, The *pre1-1* proteasome mutant<sup>14</sup> is cadmium-hypersensitive. Congenic strains expressing the wild-type *PRE1* gene and the mutated *pre1-1* allele<sup>10</sup> were plated on selection plates with and without 30  $\mu$ M cadmium and incubated for 3 d at 30  $^{\circ}$ C.

**METHODS.** Wild-type and *ubc* mutant strains are derivatives of DF5 (ref. 15). *UBC7* gene disruption was carried out by cloning the 2.2-kb *XhoI-SalI* *LEU2* carrying fragment of YEpl3 (ref. 22) into the single *HaeIII* site (at codon 43) of *UBC7*. The resulting strain (YW055) obtained by transformation, homologous recombination and tetrad analysis is MAT $\alpha$ , *ubc7::LEU2*. Other strains used in this study are YW05 (*ubc1*), YW013 (*ubc4*), YW017 (*ubc5*), YW071 (*PRE1*), YW074 (*pre1-1*), described previously<sup>10-12</sup>. Vector pSEY8 used for *UBC7* overexpression and complementation studies is a 2- $\mu$ -based plasmid carrying the *URA3* marker (M. Hall, Biozentrum, Switzerland). Standard protocols were followed for yeast techniques and media and cloning techniques<sup>23,24</sup>.

As degradation of ubiquitin-protein conjugates is mediated by the proteasome<sup>8-10</sup>, we next asked if cadmium resistance of wild-type cells requires a functional proteasome. We used a missense mutant of *PRE1*, which encodes an essential subunit of the yeast proteasome<sup>14</sup>. This mutant, *pre1-1*, is hypersensitive to amino-acid analogues and to heat shock<sup>14</sup> and fails to degrade test proteins that are rapidly degraded by the ubiquitin system in wild-type cells<sup>10</sup>. Figure 2*b* shows that *pre1-1* mutants are also cadmium-hypersensitive. This indicates that the observed cadmium resistance is mediated by ubiquitin-dependent degradation of abnormal proteins that are generated by cadmium exposure.

Key components of cellular stress-resistance pathways are typically induced in response to environmental stresses. We therefore investigated whether expression of genes involved in ubiquitin-dependent cadmium resistance increases in response to cadmium exposure. Northern analysis indicates that the levels of messenger RNA transcribed from several genes encoding

limiting components of the ubiquitin system are indeed cadmium-inducible (Fig. 3). There was strong induction with the transcript for the ubiquitin gene, *UBI4* (polyubiquitin<sup>15</sup>), and transcripts from *UBC5* and *UBC7*, whereas *UBC1* and *UBC4* transcripts were only moderately induced. Interestingly, *UBC7* transcript abundance is neither induced by heat shock (in contrast to *UBI4*, *UBC4* and *UBC5*; refs 12 and 15) nor by copper (at 200  $\mu$ M for 30 min). This suggests that the cadmium response pathway described here is not part of the general heat-shock response or the yeast copper response pathway<sup>16</sup>.

A common molecular mechanism for metal-ion resistance is the sequestration of metal ions. Animals and some yeasts (including *S. cerevisiae*) express cyteine-rich low-molecular-weight proteins, known as metallothioneins, which mediate metal resistance through their ability to chelate metals<sup>16</sup>. The phytochelatins of plants and some fungi are thought to serve similar functions<sup>17</sup>. Another mechanism for metal ion disposal is suggested by the recent finding of a putative ATP-binding-cassette type of vacuolar membrane transporter of the yeast *Schizosaccharomyces pombe* which is involved in heavy metal tolerance<sup>18</sup>. This protein may mediate metal ion resistance by pumping metal ions (or metal-peptide complexes) into vacuoles.

A complementary mechanism leading to stress resistance is the expression of specific stress proteins (such as heat-shock proteins) which act on cellular targets that are affected by the environmental challenge. Interestingly, Hsp104, which is required for tolerance to heat and other forms of stress, does not protect yeast cells against cadmium<sup>19</sup>. This suggests that most lethal lesions produced by cadmium are substantially different from those produced by heat. Our finding that cadmium resistance in yeast is mediated in part by ubiquitin-dependent proteolysis suggests that a significant part of cadmium toxicity is mediated through the formation of abnormal proteins. We propose that a notable fraction of these abnormal proteins are selectively recognized and targeted for degradation by enzymes of the ubiquitin-dependent proteolysis system. We assume that ubiquitin-conjugating enzymes (or auxiliary factors) recognize specific signals on target proteins that are created after exposure to cadmium. □

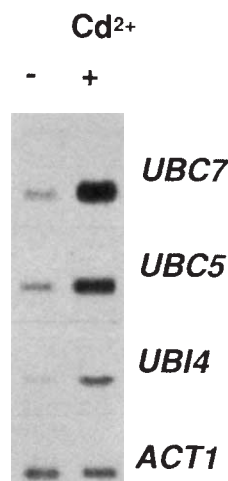


FIG. 3 Expression of the ubiquitin system increases in response to cadmium. Northern analysis of *UBC7*, *UBC5* and the polyubiquitin gene, *UBI4*. The actin gene, *ACT1*, was used as a control. RNA was isolated from exponentially growing wild-type cultures (30  $^{\circ}$ C, absorbance of unity at 600 nm) of untreated (left) and cadmium-treated (100  $\mu$ M cadmium for 30 min) cells (right). RNA (20  $\mu$ g per lane) was separated on a 1.2% agarose gel following glyoxal modification, transferred to GeneScreen (New England Nuclear) and hybridized with the corresponding probes.



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1. Stoeppler, M. & Piscator, M. (eds) *Cadmium* (Springer, Berlin, 1985).
2. Vallee, B. L. & Ulmer, D. A. *Rev. Biochem.* **41**, 91–129 (1972).
3. Finley, D. & Chau, V. A. *Rev. Cell Biol.* **7**, 25–69 (1986).
4. Hershko, A. *Trends biochem. Sci.* **16**, 265–268 (1991).
5. Jentsch, S. *Trends Cell Biol.* **2**, 98–103 (1992).
6. Jentsch, S., Seufert, W., Sommer, T. & Reins, H.-A. *Trends biochem. Sci.* **15**, 195–198 (1990).
7. Jentsch, S. A. *Rev. Genet.* **26**, 177–205 (1992).
8. Hough, R., Pratt, G. & Rechsteiner, M. *J. biol. Chem.* **261**, 2400–2408 (1986).
9. Waxman, L., Fagan, J. M. & Goldberg, A. L. *J. biol. Chem.* **262**, 2451–2457 (1987).
10. Seufert, W. & Jentsch, S. *EMBO J.* **11**, 3077–3080 (1992).
11. Seufert, W., McGrath, J. P. & Jentsch, S. *EMBO J.* **9**, 4535–4541 (1990).
12. Seufert, W. & Jentsch, S. *EMBO J.* **9**, 543–550 (1990).
13. Jentsch, S., McGrath, J. P. & Varshavsky, A. *Nature* **329**, 131–134 (1987).
14. Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C. & Wolf, D. H. *EMBO J.* **10**, 555–562 (1991).
15. Finley, D., Özkaynak, E. & Varshavsky, A. *Cell* **48**, 1035–1046 (1987).
16. Hamer, D. H. A. *Rev. Biochem.* **55**, 913–951 (1986).
17. Grill, E., Winnacker, E.-L. & Zenk, M. H. *Science* **230**, 674–676 (1985).
18. Ortiz, D. F. et al. *EMBO J.* **11**, 3491–3499 (1992).
19. Sanchez, Y., Taulien, J., Borkovich, K. A. & Lindquist, S. *EMBO J.* **11**, 2357–2364 (1992).
20. Van Nocker, S. & Vierstra, R. D. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10297–10301 (1991).
21. Phelps, A., Schobert, C. T. & Wohlrab, H. *Biochemistry* **30**, 248–252 (1991).
22. Broach, J. R., Strathern, J. N. & Hicks, J. B. *Gene* **8**, 121–133 (1979).
23. Sherman, F., Fink, G. R. & Hicks, J. B. *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, New York, 1986).
24. Ausubel, F. M. et al. (eds) *Current Protocols in Molecular Biology* (Green and Wiley, New York, 1991).

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## A new photoreactivating enzyme that specifically repairs ultraviolet light-induced (6-4)photoproducts

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CYCLOBUTANE pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts ((6-4)photoproducts) are the two major classes of cytotoxic, mutagenic and carcinogenic DNA photoproducts produced by ultraviolet light irradiation of cells<sup>1–4</sup>. The phenomenon of photoreactivation, the reduction of the lethal and mutagenic effects of ultraviolet radiation by simultaneous or subsequent irradiation with near ultraviolet or visible light, has been identified in several organisms and in some cases the enzymes that catalyse this process have been characterized in sufficient detail<sup>5</sup>. CPDs are the only known substrate for the photoreactivating enzymes so far analysed and enzymatic photoreactivation of (6-4)photoproducts has not yet been reported<sup>4,5</sup>. We report here that an enzyme that catalyses the light-dependent repair of (6-4)photoproduct exists in *Drosophila melanogaster*. This is, to our knowledge, the first report of such photoreactivating activity specific for (6-4)photoproducts in any organism.

Using the same gel shift assay as reported previously<sup>6</sup>, we detected two DNA-binding factors from *Drosophila* cell extracts, one forming a slow migrating complex (band 1) with ultraviolet-irradiated DNA and another forming a fast migrating complex (band 2) with ultraviolet-irradiated DNA. As seen in Fig. 1, by fractionation of the crude cell extract with Heparin agarose and ultraviolet-irradiated DNA affinity column chromatography, the factors forming band 1 and band 2 (factor 1 and factor 2, respectively) were separated into two fractions (lanes 3 and 5).

Factor 1 differed from factor 2 in the following ways. Depletion of CPDs from irradiated DNA with *Escherichia coli* photolyase has no effect on the binding activity of factor 1 (Fig. 1, lanes 2 and 4) but completely inhibits the binding activity of factor 2 (lanes 2 and 6), indicating that factor 1 binds to non-CPD ultraviolet damage whereas factor 2 binds to CPD ultraviolet damage. Factor 2 is a *Drosophila* photolyase (data to be published elsewhere).

Previously<sup>6</sup> we showed that band 1 disappeared from the gel shift assay after illumination of the mixture of the crude cell extract and ultraviolet-irradiated DNA with fluorescent light. To confirm this result, the ultraviolet-irradiated DNA probe T8-3 was mixed with the factor 1 fraction and followed by illumination with fluorescent light. As seen in Fig. 2a, illumination treatment resulted in disappearance of band 1 (lanes 2 and 4). To investigate whether (6-4)photoproducts are involved in the disappearance of band 1 after illumination, we used the monoclonal antibody 64M-2 which binds specifically to (6-4)photoproducts. As seen in the gel shift panel 64M-2 of

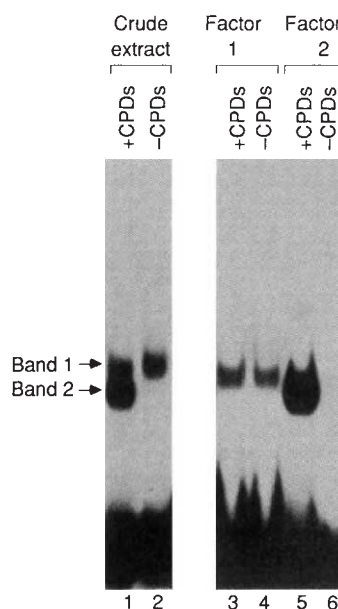
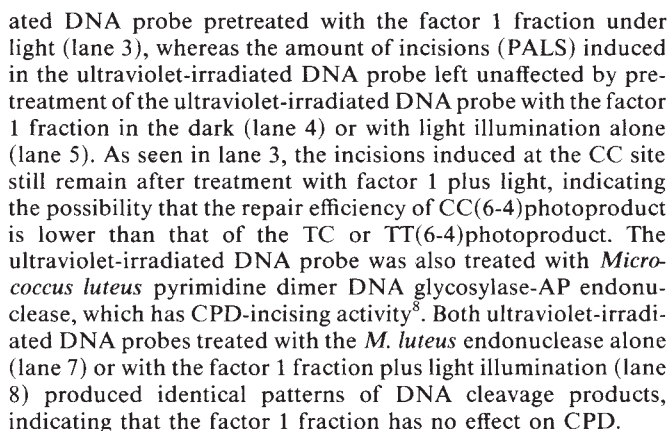


FIG. 1 Gel shift analysis showing two binding factors from *Drosophila* cells, that have high affinity for two types of ultraviolet damage, CPDs and non-CPDs, respectively. Crude cell extract (lanes 1 and 2) and the factor 1 (lanes 3 and 4) and factor 2 (lanes 5 and 6) fractions after chromatographic fractionation were assayed by gel shift for the binding activity toward an 86 base pair (bp) DNA probe, T8-3. T8-3 DNA was irradiated with 5 kJ m<sup>-2</sup> ultraviolet and used directly (lanes 1, 3 and 5) or after treatment with *E. coli* photolyase to deplete CPDs (lanes 2, 4 and 6).

METHODS. Crude extracts were prepared from embryo (Canton S) and fractionated by Heparin agarose column chromatography as previously described<sup>6</sup>. Fractions were diluted with 2 vols buffer B<sup>6</sup> and applied to the affinity column, which was prepared by coupling salmon sperm DNA after sonication, digestion with *Hinf*I and irradiation with ultraviolet at 20 kJ m<sup>-2</sup>, to CNBr activated-Sepharose 6MB (Pharmacia)<sup>14</sup>. Proteins loaded on the affinity column were eluted from the column with a linear NaCl gradient (100–1,000 mM) in buffer B. Eluted fractions showing the band 1 forming activity were pooled and reappplied to the second affinity column to obtain the factor 1 fraction. The column, from which the factor 1 fraction had been eluted out, was exposed to a fluorescent lamp (FL20SSW/18, NEC, Osaka, at a distance of 10 cm) for 5 min, and then eluted with buffer B containing 1 M NaCl, and then eluted fractions showing the band 2 forming activity were pooled as the factor 2 fraction. T8-3 DNA was prepared and used for gel shift assay as described previously<sup>6</sup>. For the photolyase treatment, 4 ng T8-3 DNA and 0.54 µg *E. coli* photolyase, which was prepared as described<sup>15</sup>, was mixed in 50 µl reaction buffer<sup>15</sup> and then exposed at 25 °C to the fluorescent lamp at a distance of 5 cm through a 2-mm-thick sheet of glass for 30 min at intensity of 12 J m<sup>-2</sup> s<sup>-1</sup> at 400 nm as monitored using a Topcon ultraviolet radiometer (Tokyo Kogaku Kikai).

**METHODS.** *a.* For treatment of the T8-3 probe DNA with the factor 1 fraction, 4 ng of end-labelled T8-3 DNA irradiated with  $5 \text{ kJ m}^{-2}$  of ultraviolet was mixed with  $12.5 \mu\text{l}$  of the factor 1 fraction in  $50 \mu\text{l}$  of the reaction buffer (2.5 mM HEPES pH 7.4, 1.25 mM  $\text{MgCl}_2$ , 4 mM KCl, 70 mM NaCl, 0.85 mM EDTA, 1 mM DTT, 0.00125% Tween20, 0.15 mM PMSF and 1.7% glycerol). The conditions for irradiation by fluorescent lamp were as in Fig. 1. The characterization of antibody 64M-2 and TDM-2 was described previously<sup>17</sup>. *b.* For PALS assay, the T8-3 probe was end-labelled with  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ . Alkaline hydrolysis at (6-4)photoproducts was done essentially as described previously<sup>18</sup>.

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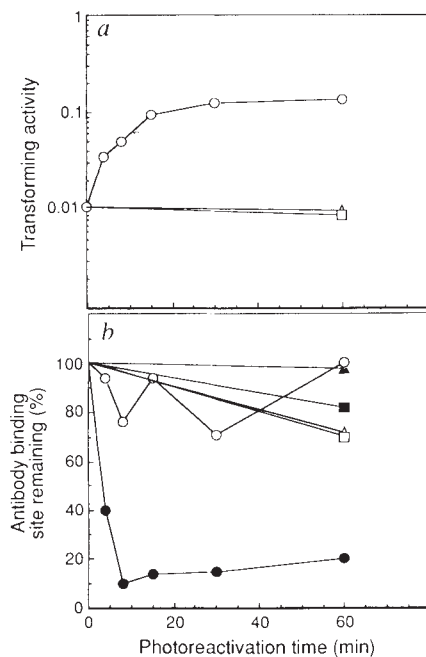
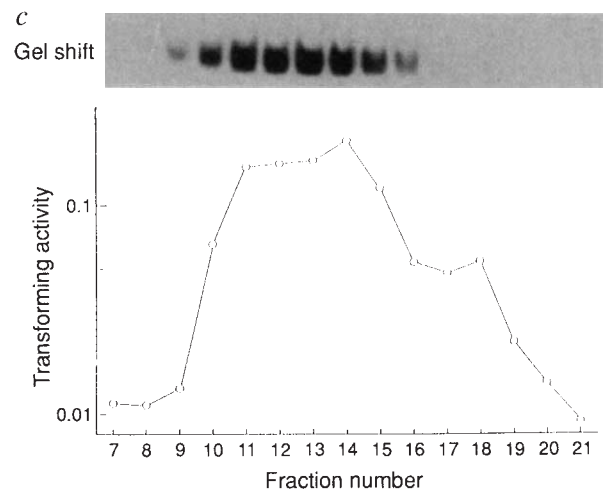


FIG. 3 Photoreactivation of transforming activity of the ultraviolet-irradiated plasmid by light-dependent modification of (6-4)photoproducts. *a*, Photoreactivation of transforming activity of the ultraviolet-irradiated plasmid. Ultraviolet-irradiated pZ189 DNA was mixed with the factor 1 fraction and exposed to fluorescent light. Aliquots of the samples were taken at various fluorescent light exposure times, phenol extracted, ethanol precipitated, treated with *E. coli* photolyase to deplete CPDs and then transformed into *E. coli* CSR603 (*phr*<sup>-</sup>, *uvrA*, *recA*); light with factor 1 (○), dark with factor 1 (△) and light without factor 1 (□). Transforming activity of each sample was calculated from the numbers of transformant colonies obtained and compared to that obtained by plasmids not irradiated. *b*, Disappearance of the binding sites for the (6-4)photoproduct-specific antibody in ultraviolet-irradiated DNA. Repair of ultraviolet damage in the photoreactivated plasmid DNA was quantified by ELISA assay using the antibody specific for CPDs (TDM-2, empty symbols) or for (6-4)photoproducts (64M-2, filled symbols).



The DNA samples and symbols are the same as those in *a* except for the omission of the treatment with *E. coli* photolyase. *c*, Coelution of the gel shifting activity and the photoreactivating activity of the factor 1 fraction. Each fraction from the ultraviolet-irradiated DNA affinity column was assayed by gel shift and transformation assay.

**METHODS.** The plasmid pZ189 was described by Seidman *et al.*<sup>19</sup>. The transformation assay was a slight modification of the method described by Sancar *et al.*<sup>9</sup>. pZ189 DNA (0.5 µg) irradiated with an ultraviolet fluence of 710 J m<sup>-2</sup> and the factor 1 fraction (100 µl) were mixed in 400 µl of reaction buffer and exposed to fluorescent light. Aliquots of the samples were removed at intervals, phenol extracted, ethanol precipitated and used for ELISA (*b*) or transformation assay in combination with the following treatment. To deplete CPDs, 30 ng samples were also treated with 0.54 µg *E. coli* photolyase, and then transformed into CSR603 cells (*a*). Details of the ELISA assay have been described elsewhere<sup>20</sup>.

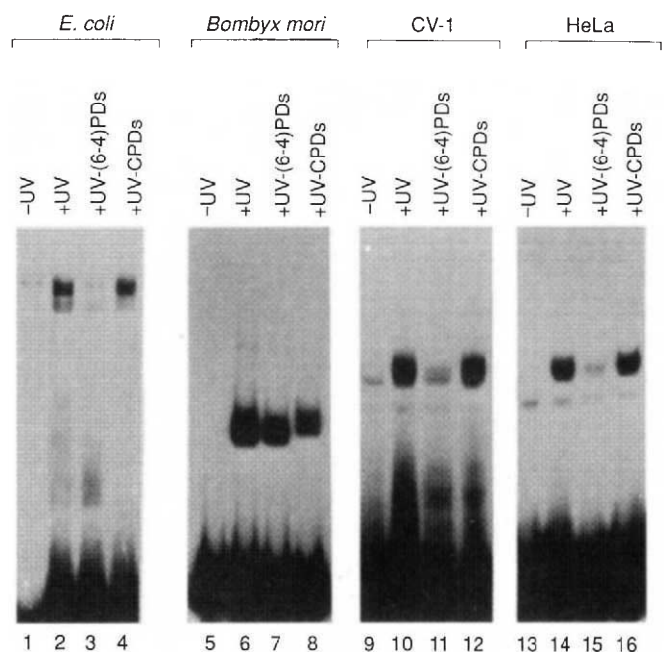


FIG. 4 Gel shift analysis of the factors which bind to (6-4)photoproduct in the extracts of *E. coli* (AB1157), silkworm (*Bombyx mori*), green monkey (CV-1) and human (HeLa) cells. Crude extracts from *E. coli* (lanes 1-4), silkworm eggs (lanes 5-8), CV-1 cells (lanes 9-12) and HeLa cells (lanes 13-16) were assayed by gel shift using T8-3 DNA unirradiated (lanes 1, 5, 9 and 13), ultraviolet irradiated (lanes 2, 6, 10 and 14), ultraviolet-irradiated but with the (6-4)photoproducts depleted using *Drosophila* factor 1 (lanes 3, 7, 11 and 15) and ultraviolet-irradiated but with CPDs depleted using *E. coli* photolyase (lanes 4, 8, 12 and 16).

**METHODS.** The nuclear extract was prepared from CV-1 or HeLa cells as described by Schreiber *et al.*<sup>21</sup>. Cellular extract from silkworm eggs was prepared according to a modification of the procedure used for the preparation of *Drosophila* extracts<sup>6</sup>.

These results support the view that the non-CPD ultraviolet damage modified by the factor 1 fraction are (6-4)photoproducts. Because the ultraviolet-irradiated DNA probe pretreated with light (Fig. 2a lanes 6 and 11, Fig. 2b lane 5) or factor 1 in dark (Fig. 2a lanes 5 and 10, Fig. 2b lane 4) had no effect on the binding of factor 1 or 64M-2 antibody and on the disappearance of PALS, we conclude that (6-4)photoproducts were not removed by direct photolysis or an enzymatic dark reaction.

To determine whether the light-dependent alteration of (6-4)photoproduct is associated with biological activity, photo-reactivation of transforming activity of ultraviolet-irradiated plasmid pZ189 treated with the factor 1 fraction was assayed using repairless *E. coli* CSR603 (ref. 9) as the host. As seen in Fig. 3a, the factor 1 fraction restored the transforming activity of ultraviolet-irradiated pZ189 DNA in a light-dependent reaction. In these DNA samples, binding sites for the 64M-2 antibody decreased with increasing time of light exposure, whereas those for the TDM-2 antibody did not change (Fig. 3b). Fractions eluted from the DNA affinity column were assayed for the band 1 forming activity and the activity to photoreactivate the transforming activity of ultraviolet-irradiated plasmid. As seen in Fig. 3c, both activities eluted as a broad but single, symmetrical peak, suggesting that factor 1 itself or co-eluting protein(s) have photoreactivating activity.

The structure of (6-4)photoproduct has already been determined<sup>10,11</sup>. In TC(6-4)photoproduct, the amino group of the 3' cytosine has been transferred to the 5 carbon of the 5' thymine. Thus, in contrast to the case of photo-reversal of CPD, (6-4)photoproducts may not be reversed to the original configuration. It will, therefore, be of great interest to see whether (6-4)photoproducts retain some alterations after photoreactivation treatment. Analysis of induced mutations in the plasmid photoreactivated with the factor 1 fraction should give an important clue to the answer.

In various organisms, DNA binding proteins that have high affinity for ultraviolet-irradiated DNA have been detected<sup>12,13</sup>. We searched for protein having specific affinity for (6-4)photoproduct among *E. coli*, silkworm (*Bombyx mori*), African green monkey cells (CV-1) and human cells (Hela) (Fig. 4). (6-4)Photoproduct-binding activities were detected in all of the extracts from *E. coli*, silkworm, monkey cells and Hela cells. When assayed for their photoreactivation-mediated activity, however, only the factor from silkworm showed positive results (data not shown). Biological functions of these (6-4)-photoproduct-binding factors, and the degree of amino-acid sequence homology between these factors remain to be determined. □

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- Harm, W. *The Biological Effects of Ultraviolet Radiation* (Cambridge Univ. Press, Cambridge, 1980).
- Friedberg, E. C. *DNA Repair* (Freeman, New York, 1985).
- Brash, D. E. *Photochem. Photobiol.* **48**, 59–66 (1988).
- Mitchell, D. L. & Nairn, R. S. *Photochem. Photobiol.* **49**, 805–819 (1989).
- Sancar, G. B. *Mutat. Res.* **236**, 147–160 (1990).
- Todo, T. & Ryo, H. *Mutat. Res.* **273**, 85–93 (1992).
- Lippke, J. A. et al. *Proc. natn. Acad. Sci. U.S.A.* **78**, 3388–3392 (1981).
- Haseltine, W. A. et al. *Nature* **285**, 634–641 (1980).
- Sancar, A., Smith, F. W. & Sancar, G. B. *J. Biol. Chem.* **259**, 6028–6032 (1984).
- Wang, S. Y. & Varghese, A. J. *Biochem. biophys. Res. Commun.* **29**, 543–549 (1967).
- Varghese, A. J. & Wang, S. Y. *Science* **160**, 186–188 (1968).
- Chu, G. & Chang, E. *Science* **242**, 564–567 (1988).
- Hirschfeld, S. et al. *Molec. cell. Biol.* **10**, 2041–2048 (1990).
- Kadonaga, J. T. & Tijan, R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5889–5893 (1986).
- Mizuno, T. et al. *Mutat. Res.* **254**, 175–184 (1991).
- Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
- Mori, T. et al. *Photochem. Photobiol.* **54**, 225–232 (1991).
- Mitchell, D. L., Brash, D. E. & Nairn, R. S. *Nucleic Acids Res.* **18**, 963–971 (1990).
- Seidman, M. M. et al. *Gene* **38**, 233–237 (1985).
- Matsunaga, T., Mori, T. & Nikaido, O. *Mutat. Res.* **235**, 187–194 (1990).
- Schreiber, E. et al. *Nucleic Acids Res.* **17**, 6419 (1989).

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## ERRATA

### Addition and subtraction by human infants

Karen Wynn

*Nature* **358**, 749–750 (1992)

THERE is an error in Table 1 of this Letter. The looking times for experiment 2, test trials of group 1–2 are reversed. As printed, the table indicates that infants' looking time LT(1) is 10.98 seconds and their LT(2) is 8.05 seconds. They should read LT(1) = 8.05 and LT(2) = 10.98. This error does not affect the conclusions of the paper, which were based on the correct looking times, not on the transposed ones.

### IPCC strategies unfair to the South

J. K. Parikh

*Nature* **360**, 507–508 (1992)

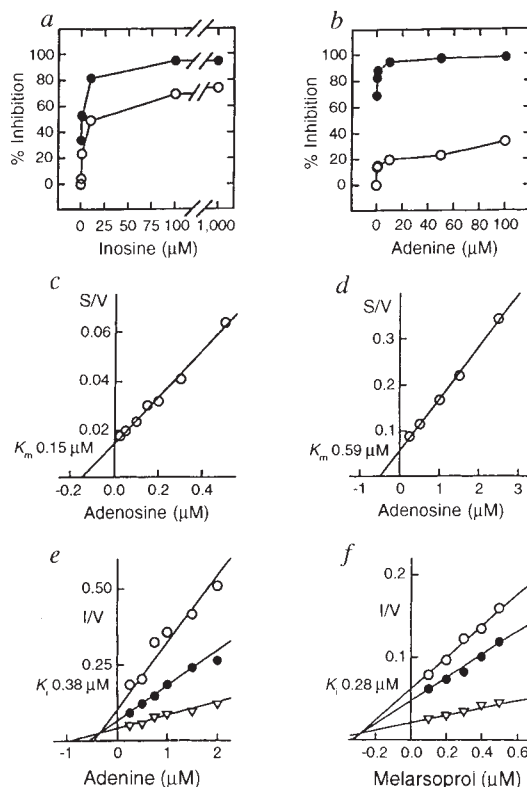
IN this Commentary article, the figures in the two left-hand columns of Table 2 were inadvertently transposed. The correct figures are 1.43 (North America); 3.05 (centrally planned Asia); 4.46 (south and east Asia) for the period 1985 to 2025 (IPCC annual growth rate). The equivalent figures for 1979–1988 (past trends) are 0.11, 4.22 and 6.77.

### Arsenical-resistant trypanosomes lack an unusual adenosine transporter

Nicola S. Carter & Alan H. Fairlamb

*Nature* **361**, 173–175 (1993)

IN some copies of the 14 January issue Fig. 3 of this Letter was poorly reproduced. It is shown here in its correct form.





# An automated approach to generating expressed sequence catalogues

Sebastian Meier-Ewert, Elmar Maier, Ali Ahmadi, Jon Curtis and Hans Lehrach

**An automated approach for partial sequence analysis of entire cDNA libraries is described with the immediate aim of generating an expressed sequence catalogue and the long-term goal of sequencing by hybridization.**

THE Human Genome Project has created a need for the development of highly parallel and automated approaches to data generation and analysis, which have been described for both genomic mapping<sup>1</sup> and sequencing projects<sup>2</sup>. We have successfully used automated procedures for generating a complete physical map of the model organism *Schizosaccharomyces pombe*<sup>3,4</sup>, a yeast. A hybridization-based approach was used to analyse arrayed genomic libraries that were spotted robotically at high density onto nylon membranes.

For genomic mapping, 40,000 clones are sufficient to give several-fold coverage of either the whole human genome in yeast artificial chromosomes (YACs), or of individual chromosomes in cosmids. However, due to the expected number of expressed sequences and their unequal representation in cDNA libraries 10–100-fold greater number of clones ( $10^5$ – $10^6$ ) are required to achieve a reasonable representation of expressed sequences within any source tissue. We have developed further automation that will allow us to extend proven hybridization-based systems to the analysis of large arrayed cDNA libraries.

## Partial sequence analysis of cDNAs

The sequence analysis of cDNA clones is a direct way to identify expressed sequences. Compared to genomic mapping, the analysis of cDNA clones requires far less DNA sequence to be analysed, although the total number of clones required is 1–2 orders of magnitude higher. cDNA analysis has so far relied mainly upon gel-based sequencing. In previously published cDNA sequencing projects<sup>5–8</sup>, typically, 200–300 base pairs are sequenced from one end of a clone ('tag-sequencing'). Although this approach generates contiguous sequence information it has several disadvantages. The sequence produced is restricted to either end of the cDNA clones. Sequencing from the 3' end of cDNA clones generates mainly 3' untranslated sequence, which while being helpful in discriminating between homologous gene family members, often gives little information about gene function. It also results in repeated sequencing of uninformative poly-(dA) stretches. Although 5' sequencing can overcome these limitations, varying clone lengths mean that sequence

information may not be generated from the same position in homologous clones and true identity may be missed. In addition, tag-sequencing is labour intensive and expensive.

Hybridization-based partial sequence analysis is an alternative method for generating sequence information of cDNA clones<sup>9</sup>. As in genomic mapping approaches based on hybridization, tens of thousands of cDNA clones can be analysed in parallel. The system is based on the statistical probability of sequence identity of clones sharing a given number of hybridization events. Short oligonucleotides (7–9-mers) are used as hybridization probes. The results of 100–200 8-mer hybridizations, which can be performed within eight weeks, produce a characteristic fingerprint of matching and non-matching oligonucleotides that can discriminate over 100,000 sequences. Because the fingerprint is a collection of nearly random sampling events, the sequence information generated for each clone is distributed evenly along its length, avoiding the above mentioned limitations of tag-sequencing.

The purity of target DNA required for the sequence-specific hybridization of short oligonucleotides is far higher than for genomic mapping. For this reason a high-throughput DNA purification procedure is essential for the analysis of entire cDNA libraries.

Here we present a system for large-scale analysis of cDNA libraries with the aim of creating a catalogue of all expressed sequences within an organism (Fig. 1).

## 384-well plates

The ever increasing number of arrayed libraries generated has presented an acute plate storage and handling problem, especially in view of the expansion to arrayed cDNA libraries that contain in the order of

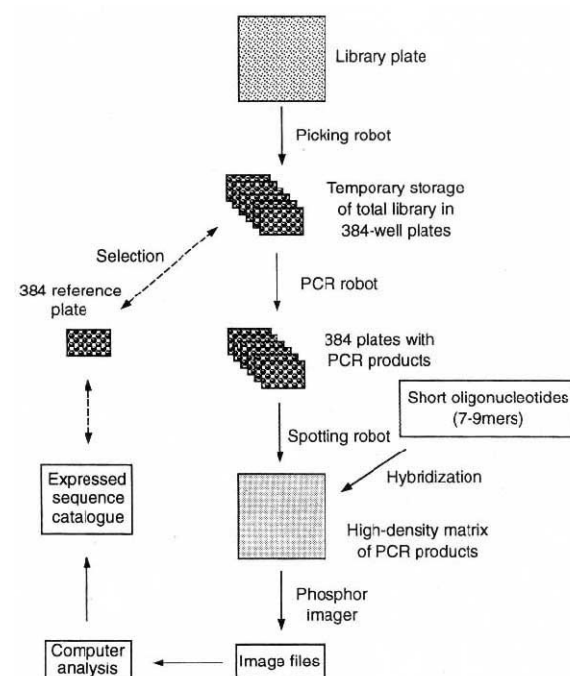


FIG. 1 Scheme of an automated approach to generating expressed sequence catalogues.

$10^5$ – $10^6$  clones each. To address this problem a commercially available microtitre plate has been designed that contains 384 wells ( $4 \times 96$ ) (Genetix Ltd, Christchurch, UK), with identical external dimensions to conventional 96-well microtitre plates. The volume of 70  $\mu$ l per well means that the plates are suitable for culturing both *Escherichia coli* and yeast cells, which grow to a density of  $A_{600} = 0.25$  and  $A_{600} = 2$ , respectively. Conventional plate shakers can be used to resuspend settled cultures after prolonged storage. These higher capacity microtitre plates represent a considerable saving in terms of handling and storage of clones.

## Picking robot

Arrayed randomly plated clones into storage microtitre plates is a labour-intensive and time-consuming process. A skilled person can pick up to 4,000 clones per day manually. To be able to array routinely large cDNA libraries with 100,000 clones each it becomes necessary to automate this

process. We have therefore developed a new automated colony picker offering some improvements over earlier designs<sup>10,11</sup>. Up to 10,000 clones can be picked with a 96-pin head directly into 384-well plates in one session of about 5 h without operator input.

### High-throughput PCR procedure

The hybridization of short oligonucleotides (7–9-mers) requires the recombinant target DNA to be purified away from host-cell DNA to prevent clone-specific signal from being obscured by background noise. The most efficient way to purify cDNA from *E. coli* on a large scale is to perform vector-primed polymerase chain reactions (PCRs). PCR is performed directly in polypropylene 384-well plates, using a specially developed heat-sealing system (Genetix), by cycling the plates between waterbaths. A PC-controlled robotic arm with a basket carrying up to 120 plates is cycled between large watertanks maintained at required temperatures (Fig. 2). Direct inoculation of *E. coli* or yeast cells into PCR mixtures can be used to amplify specifically recombinant DNA. This is done with a commercially available 384-pin transfer device (Genetix). Using this system up to 46,000 PCR reactions can be performed by a single person in one day. The high specificity and yield of the reaction makes the PCR products ideal hybridization targets.

### Automated clone analysis

In order to analyse clones by hybridization the DNA is immobilized on solid supports. The use of arrayed genomic clone libraries, and also to a limited extent arrayed cDNA libraries, has previously been reported from our laboratory<sup>12</sup>. We have developed a new spotting robot that is largely based on the

design of an earlier machine successfully used in the laboratory for several years. The new spotting robot has a greatly increased throughput to allow the use of large arrayed libraries. Typically, 36,000 clones are spotted onto each of twelve 22 cm × 22 cm filters in 2 h.

Filters hybridized with radioactively-labelled oligonucleotides are then scanned using phosphor storage screens and the image captured using a phosphor imager (Molecular Dynamics, Sunnyville, California). Software already developed for genomic mapping projects (R. Mott, *et al.*, submitted) has been adapted in collaboration with Jens Michael Carstens of the Technical University of Denmark to assign automatically grid locations to each hybridization signal and store the results in a relational database.

### Sequencing by hybridization

The automation procedures presented here fulfill many of the technical requirements for sequencing by hybridization, which has emerged as an alternative to gel-based methods in the past few years<sup>12–14</sup>. We are now focusing on the two remaining areas in which further developments are necessary. Firstly, the automation of short oligonucleotide hybridization to generate datasets two orders of magnitude larger than for partial sequence analysis and secondly the development software for sequence reconstruction from hybridization results. The technology is however already sufficiently developed to allow sequence fingerprinting of a large number of cDNA clones.

### Expressed sequence catalogue

By generating sequence fingerprints of cDNA libraries constructed from poly(A)<sup>+</sup> RNA derived from different tissues or different developmental stages of the same tissue, expression data can be obtained on a large set of sequences. The more clones are analysed from each library the more rarely expressed sequences can be characterized. In our present system it is realistic to aim for 100,000 clones per library, which should allow the identification of most common or moderately expressed sequences. Further development of the analysis systems, particularly towards miniaturization of clone arrays and the use of non-radioactive detection systems that have higher resolution, could make it possible to generate sequence fingerprints of one million clones per cDNA library. At present, there are cDNA libraries in production from ten human

fetal tissues. It will not be necessary to store permanently all these clones, as after sequence finger-printing only one clone, or in the case of splice variants a small number of clones, for each sequence need be retained. Eventually a sub-library can be assembled of all the DNA sequences expressed in a wide variety of tissues at various developmental stages, thus generating an expressed sequence catalogue of an organism (Fig. 1). In the case of the human genome the number of sequences is likely to be of the order of 100,000.

Such an expressed sequence catalogue would represent a major resource in large-scale genome analysis, allowing both physical map assignment and efficient sequencing of a large proportion of genes in any given genome. □

Sebastian Meier-Ewert, Elmar Maier, Ali Ahmadi, Jon Curtis and Hans Lehrach are in the Genome Analysis Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, PO Box 123, London WC2A 3PX, United Kingdom. For more information, fill in reader service number 100.

1. Bellanne-Chantelot, C. *et al. Cell* **70**, 1059–1068 (1992).
2. Endo, I., Soeda, E., Murakami, Y. & Nishi, K. *Nature* **352**, 89–90 (1991).
3. Maier, E. *et al. Nature Genet.* **1**, 273–277 (1992).
4. Hoheisel, J. D. *et al. Cell* (in the press).
5. Adams, M. D. *et al. Science* **252**, 1651–1656 (1991).
6. Adams, M. D. *et al. Nature* **355**, 632–635 (1992).
7. Okubo, K. *et al. Nature Genet.* **2**, 173–179 (1992).
8. Kahn, A. S. *et al. Nature Genet.* **2**, 180–185 (1992).
9. Drmanac, R. *et al. in Electrophoresis, Supercomputing and the Human Genome* (eds, Cantor, C. & Lim, H. A.) 60–74 (World Scientific, Singapore, 1990).
10. Ueber, D. C., Jaklevic, J. M., Theil, E. H., Lishanskaya, A. & McNeely, M. R. *BioTechniques* **5**, 642–645 (1991).
11. Jones, P., Watson, A., Davies, M. & Stubbings, S. *Nucleic Acids Res.* **20**, 4599–4606 (1992).
12. Lehrach, H. *et al. in Genome Analysis: Genetic and Physical Mapping* (eds, Davies, K. E. & Tilghman, S.) 36–81 (Cold Spring Harbor, New York, 1990).
13. Drmanac, R., Labat, I., Brukner, I. & Crkvenjakov, R. *Genomics* **4**, 114–128 (1989).
14. Strezoska, Z. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 10089–10093 (1991).

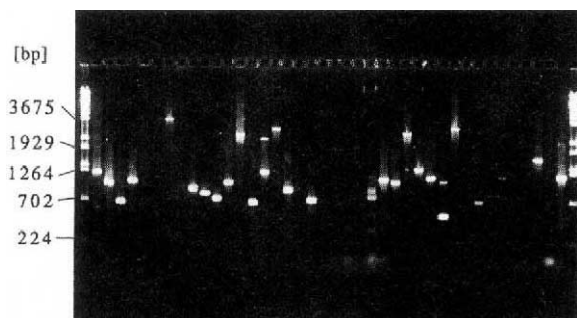


FIG. 2 PCR amplification of cDNA clone inserts. cDNA was cloned into the *Sal*/*Not*I digested vector pSPORT. cDNA was amplified using vector primers flanking the cloning site; Sport/3 20-mer: 5'-CCGGTCCGGAATCCCGGGT, Sport/5 29-mer: 5'-GCACGCGTACGTAAGCTTGGATCCTCTAG. Amplification was carried out in 384-well microtitre plates in a volume of 30 µl containing: 10 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl pH 8.55, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatine, 2 mM dGTP, 2 mM dCTP, 2 mM dATP, 2 mM dTTP, 0.5 units *Taq* polymerase. The sealed 384-well microtitre plates were cycled automatically 30 times between waterbaths at 96 °C for 3 min and 73 °C for 5 min. 5 µl of 40 reactions were analysed on a 1% agarose gel using *Bst*FI digested λ DNA as size standard. Of 200 reactions analysed, 75% contain product visible by ethidium bromide staining and 5% contain more than one product.

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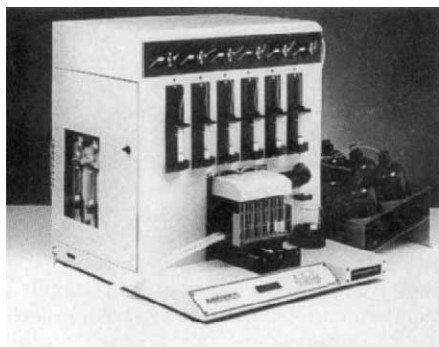
Reader Service No.56



# Mechanized means

Featured this week are products designed to automate laboratory tasks, including an instrument for processing western blots, an infrared fluorescence-based sequencer and robotic liquid handling systems.

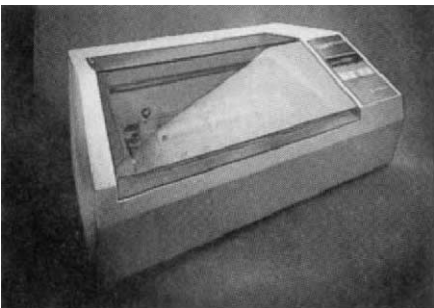
The AutoTrace SPE workstation for water analysis from Zymark has been designed to automate the time-consuming and often error prone steps in solid-phase extraction (SPE) of trace contaminants from water (*Reader Service No. 101*). The company says that it can be used to process over 24 one-litre water samples in an 8-h shift,



AutoTrace water analyser.

requiring only 15 min of operator time. Through the use of precision syringes, pumps and menu-driven software, the AutoTrace processes up to six water samples simultaneously. Sample volumes can range from as low as 10 ml to as high as 2 l. Five reagents can be used for conditioning and elution steps. The workstation uses 1-, 3-, 6- or 8-ml syringe barrel SPE cartridges, which can be dried with an inert gas, if desired. To make solvent disposal easier and less costly, the waste stream separates the water and organics. The conditioning, sample delivery and elution steps are microprocessor-controlled. The unit runs with its own internal operating system — a dedicated computer is not necessary.

The MAPS (Membrane Assay Processing System) instruments from BioGenex Laboratories are intended to simplify and expedite western blot processing, ensuring standardized results and facilitating safe

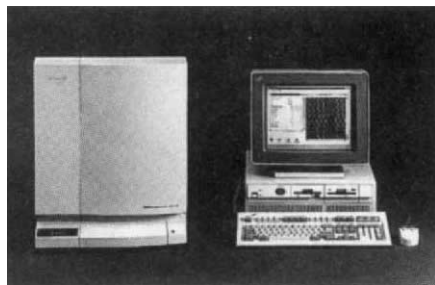


BioGenex's western blot processor.

waste disposal (*Reader Service No. 102*). All MAPS processors feature a closed-system design that is designed to minimize exposure to infectious materials. The instrument can process either strip assays with the WesStrip module or sheet assays with the WesPage module; these modules accept strip and sheet assays from most manufacturers. As a standalone processor, the MAPS instrument will run preprogrammed protocols. Alternatively, a PC-compatible software interface allows the user to custom design protocols. With these protocols, the MAPS processor automates washing and incubation cycles and alerts users for the addition of samples and detection reagents. Two MAPS models are available for *in vitro* diagnostic use: MAPS I processes up to 20 strip tests or 48 test sheets; MAPS II can accommodate 40 strip tests or 96 sheet tests. Both models are available for confirmatory testing of sera samples. BioGenex also offers strip and sheet format western blot test kits for the detection of antibodies to HIV-1 or HTLV-1 in human sera.

## ■ DNA synthesis/sequencing

The new Model 4000 DNA sequencer from LI-COR is an infrared fluorescence-based sequencer that automates the electrophoretic separation and base calling of DNA fragments (*Reader Service No. 103*). DNA



LI-COR's Model 4000 automated, infrared fluorescence-based DNA sequencer.

primers are labelled with a novel, infrared fluorescent dye, which provides good sensitivity for detection, LI-COR says. A solid-state laser diode is used to excite the labelled fragments and the infrared emission is detected with a silicon avalanche photodiode. The use of solid-state detection components is intended to increase reliability, while reducing operating and maintenance costs and space requirements. LI-COR says that up to 11 samples can be sequenced within 7 h of sample loading. The Model 4000's Base ImagIR software presents data in real time during electrophoresis using an image format like an autoradiogram. Base ImagIR's

SmartStep and AutoStep sequencing software uses the image to sequence over 500 bases per sample with 99 per cent accuracy, according to LI-COR. This multitasking software also allows two additional sequencers to be operated by the same computer, which is designed to reduce the cost of future expansion. For flexibility, the Model 4000 can use gels of various heights, thicknesses and composition, as well as a variety of combs and lane-loading formats.

Applied Biosystems says that automated DNA/RNA synthesis is made easier with AutoAnalysis, a new feature added to the company's Model 392 medium-throughput and Model 394 high-throughput DNA/RNA synthesizers (*Reader Service No. 104*). The new AutoAnalysis enhancement makes synthesizing PCR primers simpler and more cost-effective, says the company. It includes a trityl monitor that automatically measures the level of DMT-protecting group released during detritylation. ABI says that it eliminates the need for a fraction collector and the worry of collecting, analysing or disposing of trityl samples. The synthesizer constantly monitors the synthesis and displays the results. If an oligonucleotide fails to meet the prespecified coupling efficiency anytime during synthesis, the synthesizer automatically stops operating, preventing wasteful consumption of expensive reagents. AutoAnalysis is included on the Model 394 and is optional on the Model 392 synthesizer.

Genetix, in collaboration with the Imperial Cancer Research Fund (ICRF), has developed a new system for the storage and thermal cycling of small genomic samples (*Reader Service No. 105*). The Q-System products are a range of precision-moulded plastic consumables based upon a quadrupole density (384 well) multiwell Q-plate format and have been designed to fulfil the increased throughput requirements of genome products worldwide. The individual wells, 70 µl in volume, are spaced to maintain compatibility with all available pipetting devices. Genetix says that their use can result in freezer space being reduced by over 400 per cent. Included in the range is a 384-pin replicating device for manual or robotic sample transfer or direct spotting onto filter membranes. In a further collaboration with ICRF, Genetix now produces a hermetically sealable 384-well microplate for thermal cycling. The patented process that seals each 70-µl well utilizes a novel polymer laminate film that is welded onto

## PRODUCT REVIEW

the plates using the company's own heat-sealing machine. The complete system is intended for either the long-term storage of genomic libraries, small sample transport or direct thermal cycling studies. In the later case, sequencing can be achieved without the need for oil overlays on sample volumes as low as 5  $\mu$ l, greatly reducing consumption of polymerase enzyme. Genetix will be producing a thermal cycling system based on the quadrupole density plate that can handle in excess of 50,000 sequencing samples simultaneously.

### ■ Benchtop aids

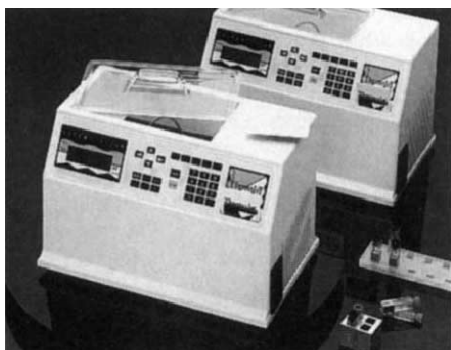
Camag's automatic developing chamber for thin-layer chromatography offers complete control of the developing conditions from plate to plate (*Reader Service No.*



Camag's automatic TLC developing chamber.

106). The operator can select the parameters: whether to precondition the tank or not, tank or sandwich configuration, running distance and plate drying conditions. Developing programs can be stored for easy recall and use.

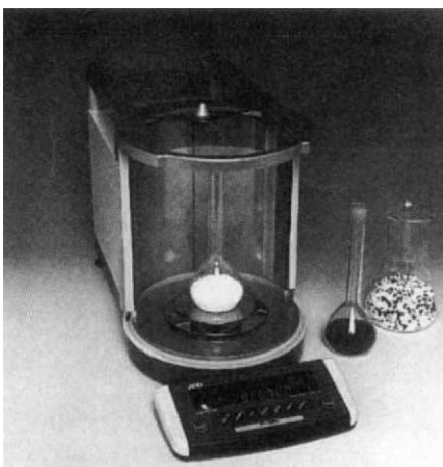
The new Thermojet thermal cycler from EquiBio can be programmed in three ways: from the integral keyboard and interactive display, from the internal memory or by means of Smart Cards (microchip cards of less than 1-mm thick containing eight programs per card), which are designed to



EquiBio's Smart Card operated Thermojet thermal cycler.

provide an easy way of storing, filing and reproducing programs (*Reader Service No. 107*). Smart Cards are designed to avoid the danger of entering programs incorrectly or of discovering undesired changes in a program. Thermojet units do not require water cooling, instead they use heavy-duty, 150°-rated proprietary Peltier modules. Using a Peltier module that is the same size as the blocks should ensure high precision and uniformity of temperature in every well: adjustments from  $-9.5$  to  $99^{\circ}\text{C}$  are controlled to better than  $0.5^{\circ}\text{C}$ , EquiBio says. The well temperature of Thermojet can also be monitored by a probe tube when the operator wants better to appraise the temperature lag between block and tube. With this additional tool, the operator can derive the information that is needed to optimize the process, to compare the performance of tube models or to solve various problems. Units are available for sixteen 0.5-ml tubes or for 36 200- $\mu$ l tubes, and for all voltage requirements.

The most striking feature of the HA-200A balance from A & D Instruments is the cylindrical and pillarless weighing chamber (which has been motorized) and the new display pod, which incorporates many

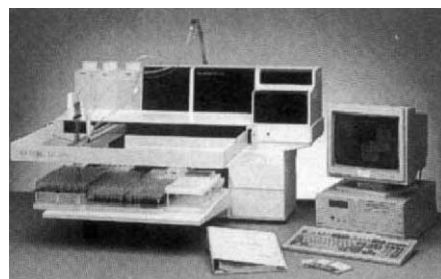


A & D Instruments' new cylindrical balancing act.

features from the HX Series (*Reader Service No. 108*). The weighing chamber has three circular side doors, each of which will rotate to open any amount and any degree (permitting the user  $200^{\circ}$  access), and an additional top entry door. Two models are available: the HA-M balances where the doors are opened manually and the HA-200A with tinted glass side doors that are automatically controlled. Other features include a dual-colour detachable display, new keyboard design, fully automatic self-calibration, and an increased weighing capacity that has been expanded to 210 g. New weighing functions have been introduced, including counting, per cent and comparator mode. Optional features that were previously unavailable include analog output and full control by a remote commander.

### ■ Liquid handling systems

The new Quatro SP-400 high-speed, four-probe robotic sample processor from Quatro Biosystems has a capacity of up to 960 17-mm diameter tubes or twenty 96-well microplates and is designed for larger volume assays and routine screening in clinical laboratories (*Reader Service No. 109*). The company says that the accuracy

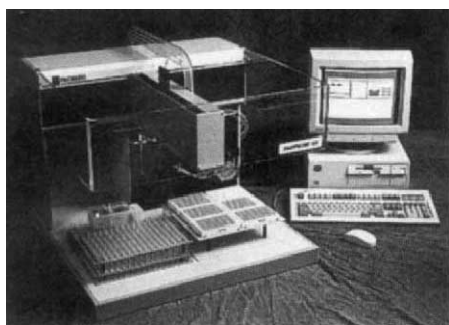


High-speed sample processor — the SP-400.

and precision of sample and reagent delivery is ensured by independent liquid-level sensors linked to the high-resolution syringe unit. Probes are also available with an internal and external Teflon coating. This reduces carryover to 0.0002 per cent, Quatro Biosystems says. Using the hand-held controller and resident EPROM software, the SP-400 can be programmed for a variety of liquid handling requirements, including aspirations from individual standard or reagent bottles. For more demanding protocols, the workstation can be programmed and controlled via an external personal computer using the company's Concerto software. The SP-400 includes the new Modulex workstation system, which is designed to improve and simplify significantly the location of racks and containers, ensuring that they remain securely in their programmed positions. Operators can also mark out the position of their racks and containers in a choice of colours to speed up and simplify workstation assembly. Available options include an automatic sample transport system with integral laser bar-code reader. This reader reads each tube as it moves into the sampling position to ensure positive patient identification.

MultiPROBE, the robotic four-tip liquid handling system from Packard is designed to process over 1,200 samples per hour (*Reader Service No. 110*). The company says that the key to the new multitipped liquid handling system is the multichannel sample processing technology featuring Varispan. Unlike fixed-tip systems, Varispan enables the operator to vary the spacing between sampling tips. This set-up enables any combination of tubes, plates and vials to be processed without reducing throughput. Carryover is eliminated with high-pressure, high-volume washing, not traditional timely syringe washing. Liquid-level sensing specifications, once unique to single-tip handling

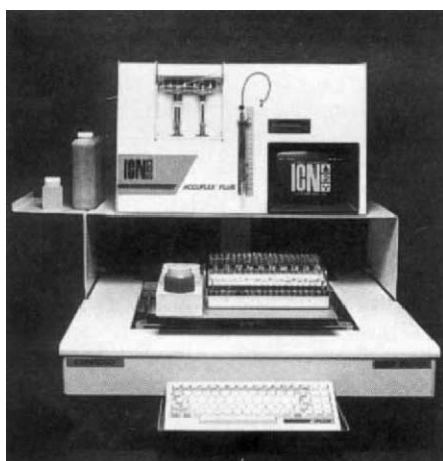




**MultiPROBE robotic system.**

systems, are now available on a multitip system through Packard's Accusense technology. User prompts and an easy-to-use graphical interface facilitates use of the software.

ICN Biomedicals has a **liquid delivery system** called the Accuflex Plus that is designed to simplify even the most difficult to automate procedures (*Reader Service No. 111*). The system should find application in a variety of areas: radioimmunoassay procedures, including double antibody, coated tube and magnetic separation; EIA, ELISA and IRMA procedures, which can be run in tubes, microplates and bead trays; dilutions for RID testing; column affinity chroma-

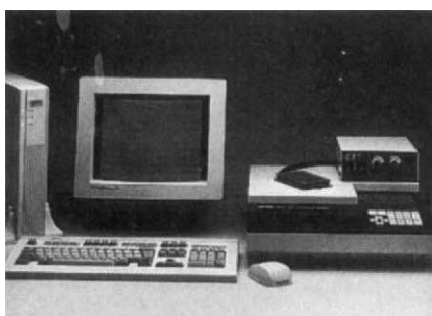


**Accuflex Plus liquid delivery system.**

tography, including sample loading, elutions with various solvents and separate eluent collection; protein crystallization by the hanging drop method; screening for HIV, hepatitis and drugs of abuse using bead trays, microtitre plates and test tubes; and reagent dispensing of small volumes, precious or hazardous liquids. The Accuflex Plus offers a wide variety of reagent containers, racks and carriers.

### ■ Detection systems

Bio-Rad's new Model 3550-UV **microplate reader** offers the broad range of functions required for the wide variety of assays now being routinely performed in the laboratory (*Reader Service No. 112*). In addition to enzyme immunoassays, almost any test system that produces a chromogenic or



**Multifunctional 3550-UV microplate reader.**

light-scattering result can be analysed on the new microplate reader. Protein determination, cell viability, DNA detection, NAD/NADH assay, kinetic ELISA and kinetic enzyme analysis are just a few examples of the growing list of routine assays being transferred from the cuvette to the microplate format. The user-friendly software allows the user to create a custom format, activate the plate mixer, select the reports and set reading delays and reading intervals. All of this information comprises a customized analysis protocol, up to nine analysis protocols and nine user-programmed formats can be permanently stored for fast retrieval. The 80 × 16 LCD allows the user

to view a complete set of data, an entire plate format or a whole analysis protocol on a single screen. The 3550-UV reader offers a spectral range of 340–750 nm and new high-performance optics, which increases linear range to  $A = 3.0$ . A modular design format allows the addition of an automatic stack-loading mechanism with bar-code reader. This set-up converts the instrument to a multiple plate reader, which is capable of reading 25 plates in about 12.5 min. A dual-channel microplate incubator can also be installed and ensures thermal regulation to both the pipetting and reading stage of microplate analysis.

The new Shimadzu AA-6501 **atomic absorption spectrophotometer**, which is supplied by V. A. Howe, comes in four different systems, from a basic flame to a fully automated unit (*Reader Service No. 113*). There are three features of this series that may be of interest to users: microsampling and automatic sample pretreatment, a choice of background correction, and automatic switching and align-



**From basic flame to a fully automated unit — see the Shimadzu AA-6501 system.**

ment between the flame burner head and graphite furnace. A single 'intelligent' preparation system can perform standard curve dilutions from a single stock standard, sample pretreatment with up to six reagents, automatic sample dilution for out-of-range samples, matrix modification, automatic

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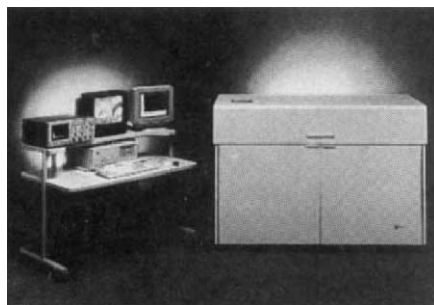
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injection to flame or furnace and sequential analysis for up to 12 elements. As well as conventional  $D_2$  background correction traditionally used to overcome interferences from molecular absorption, particulate scattering and chemical interferences, the AA-6501 also incorporates the high-speed self-reversal background correction technique. This is particularly important to eliminate spectral interferences and absorption line overlapping, as well as chemical, particulate and molecular interferences. The advantages of the self-reversal method are: only one lamp is used to measure both atomic absorption and background absorption, the technique is suitable for trace component analysis in matrices showing complex background absorbances (that is, biological samples or alloys) and background correction is possible over the whole wavelength range (190–900 nm). A patented Shimadzu single-body dual-atomizer system is designed to ensure optimum performance and ease of use for automated atomic absorbance. This system will automatically change from flame to furnace and adjust the optical alignment of the unit in three dimensions to ensure reproducible and optimal sensitivity. Coupling this to the eight lamp turret and user-defined macro-programming will produce 12-element sequential analysis.

Vestec is announcing a fully automated ResearchH **matrix-assisted laser desorption time-of-flight mass spectrometer** for determining accurate molecular weights on biopolymers (*Reader Service No. 114*). This instrument is engineered for rapid sample throughput and routine use. In addition to a video sample monitor for viewing each sample and a joystick for fast manual operation, the LaserTec product line includes complete automation for controlling laser intensity and sample position on the 24-sample carousel. This 'intelligent' automation makes decisions about optimum laser intensity and sample position, allowing the Vestec instrument to operate unattended. The LaserTec ResearchH is suitable for the



**Automation for the LaserTec ResearchH TOF mass spectrometer.**

automatic analysis of a wide range of biomolecules and is designed with the research scientist in mind. It is supplied in a desk-size cabinet and comes standard with a 1.2-m linear flight tube and a nitrogen laser. The LaserTec ResearchH is engineered to accommodate additional lasers and can be configured with several access ports and/or a high-performance reflector.

Flow-Count, a new line of **radio-chromatography flow detectors** from Bioscan, combines the flexibility of remote operation with accurate counting of gamma, positron and high-energy beta emitters over a range of  $10^2$ – $10^{12}$  d.p.m. (*Reader Service No. 115*). Interchangeable detectors, including NaI scintillators and miniature PIN diode detectors, are easy to position and shield in the hood, 'hot-cell', near a UV detector or in any other tight space up to 12 feet from the compact base unit, Bioscan says. Disposable flow cells avoid contamination and are easily prepared from standard tubing. Flow-Count is compatible with all recorders, integrators and chromatography data systems. Interfacing Flow-Count with Bioscan's Quick-Count creates a comprehensive system with capabilities that include pulse counting, peak integration, graphics printer and RS232C output. Flow-Count should find application in monoclonal antibody research, protein labelling and purification, in addition to nuclear medicine and PET.

Photon Technology has opened up new areas of application with the launch of the company's ImageMaster series of quantitative **ratio fluorescence imaging systems** specifically designed for spatial resolution of intracellular ion concentrations (*Reader Service No. 116*). This new development combines the speed and powerful data handling capabilities of the presently available 486-based ELISA PC, the performance and features of the 640 image processing board and the application software programs of PTI. By asking PTI to add the camera and microscope of your choice, it is possible to tailor a system to suit your own specifications. There are over a dozen standard ratio fluorescence imaging systems and a wide range of options, including high-speed (millisecond), dual-channel excitation and emission photometry. The open architec-

ture design of the imaging systems means that they can easily be modified and upgraded. PTI offers short courses on ratio fluorescence imaging techniques if you feel a need to brush up on this area.

Solid-state low concentration and high concentration **UV-based ozone monitors** from Ozone Research and Equipment automatically measure ozone in air or oxygen at levels from 0 to 1,000 p.p.m. by volume, or 0–100 mg  $sL^{-1}$  weight (*Reader Service No. 117*). These monitors feature digital readout, alarm and control capability, and incorporate standard design features including automatic temperature compensation, pressure compensation, rapid warm-up and real-time, online monitoring. OREC Hi-Con monitors are typically used to measure the ozone output of an ozonator, or to measure ozone off-gas downstream from an ozone contacting system to provide feedback and control the output of the ozonator. This ensures tight process control and minimizes energy consumption. Lo-Con monitors, on the other hand, are used to measure ambient ozone levels and can be interlocked with the ozonator via an optional alarm to shut the system down and start an exhaust fan in the event of ozone detection above a specified level.

Gould Electronics has introduced a **high-voltage oscilloscope probe**, Type PB49, which allows safe working when making measurements with differential inputs (*Reader Service No. 118*). The new probe is intended for use with instruments such as the Gould 1600 Series family of digital storage oscilloscopes, which incorporate both single-ended and differential inputs. With the probe, the instrument can be used for off-ground measurements at high voltages, while still meeting the 'safety at work' requirements by maintaining an earth connection to the oscilloscope at all times. The PB49 probe, which has an impulse withstand voltage of 3.2 kV, meets the Category I, II and III overvoltage specifications laid down by the International Standards Organization for use on single- and three-phase mains-voltage systems.

The X-MET 820 from Outokumpu can be used to **analyse up to six different elements simultaneously** by energy dispersive XRF (*Reader Service No. 119*). It can be fitted with one or two probes (with a Fe, Cd, Cm or Am source). Therefore, the instrument is capable of analysing all the elements from aluminium to uranium. There can be 24 different programs (calibration models) at a time. The X-MET 820 provides analyses from liquid, powder or solid samples. □

*These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.*

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